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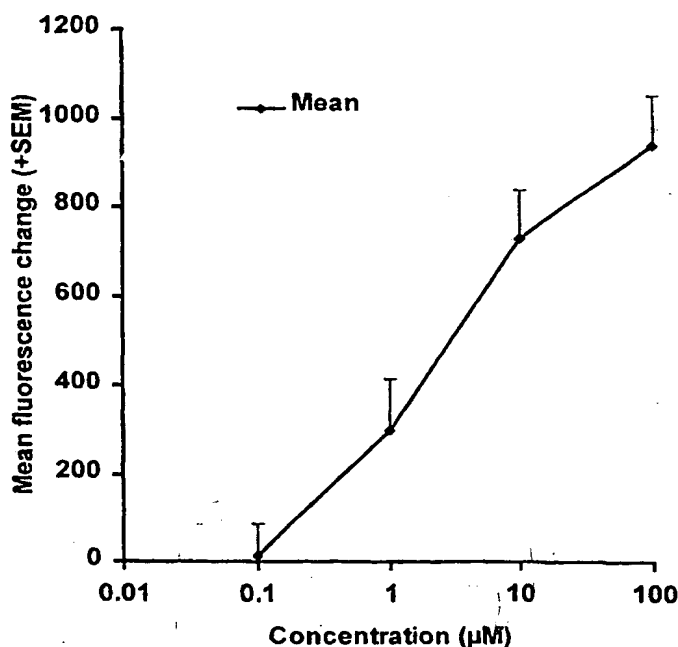
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(54) Title: AN IMPROVED METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR RESPONSE

## (57) Abstract

An improved method and tools for quantifying the effect of an influence on cellular response is described. In particular, an improved method is described for detecting intracellular translocation or redistribution of biologically active polypeptides. The invention also describes several ways of contacting the cells with a substance influencing a cellular response and extracting quantitative information relating to the response in a highly parallel fashion. The method may be used as a very efficient procedure for testing or discovering the influence of a substance on a physiological process using commercially available parallel, high volume assay techniques, for example in connection with screening for new drugs, testing of substances for toxicity, and identifying drug targets for known or novel drugs.

hPKC $\beta$ 1-GFP ATP dose-response  
in FLIPR (n=6)



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## AN IMPROVED METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR RESPONSE.

### SUMMARY OF THE INVENTION

- 5 The present invention relates to an improved method and tools for extracting quantitative information relating to an influence on a cellular response, in particular an influence caused by contacting or incubating the cell with a substance influencing a cellular response, wherein the cellular response is manifested in redistribution of at least one component in the cell. In particular, the invention relates to an improved method for
- 10 extracting the quantitative information relating to an influence on an intracellular pathway involving redistribution of at least one component associated with the pathway. The method of the invention may be used as a very efficient procedure for testing or discovering the influence of a substance on a physiological process, for example in connection with screening for new drugs, testing of substances for toxicity, identifying
- 15 drug targets for known or novel drugs. In particular, the present invention relates to an improved method for parallelisation of the testing procedure so that a large number of substances can be tested simultaneously using commercially available instrumentation. The invention also describes several ways of contacting the cells with a substance influencing a cellular response and modifications made to the actual cells before, during
- 20 or after contacting the cells with these substances as to improve the applicability and use of the method for extracting quantitative information relating to influence on an intracellular pathway in a highly parallel fashion. Other valuable uses of the method and technology of the invention will be apparent to the skilled person on the basis of the following disclosure. In a particular embodiment of the invention, the present invention
- 25 relates to a method of detecting intracellular translocation or redistribution of biologically active polypeptides, preferably an enzyme, affecting intracellular processes, and a DNA construct and a cell for use in the method.

### BACKGROUND OF THE INVENTION

- 30 Intracellular pathways are tightly regulated by a cascade of components that undergo modulation in a temporally and spatially characteristic manner. Several disease states can be attributed to altered activity of individual signalling components (i.e. protein

kinases, protein phosphatases, transcription factors). These components therefore render themselves as attractive targets for therapeutic intervention.

Protein kinases and phosphatases are well-described components of several  
5 intracellular signalling pathways. The catalytic activity of protein kinases and phosphatases are assumed to play a role in virtually all regulatable cellular processes. Although the involvement of protein kinases in cellular signalling and regulation have been subjected to extensive studies, detailed knowledge on e.g. the exact timing and spatial characteristics of signalling events is often difficult to obtain due to lack of a  
10 convenient technology.

The measurement of the activity of intracellular enzymes, such as kinases and phosphatases, can be performed by well-established procedures, both manually and in various automated forms, at throughput rates which make these measurements useful in  
15 the search for new drug candidates. In addition to measures of activity, measures of the distribution of these and other enzymes in the cell has proven useful, and established techniques exist for this type of measurement as well. Protein kinases often show a specific intracellular distribution before, during and after activation. Monitoring the translocation processes and/or redistribution of individual protein kinases or subunits  
20 thereof is thus likely to be indicative of their functional activity. A connection between translocation and catalytic activation has been shown for protein kinases like the diacyl glycerol (DAG)-dependent protein kinase C (PKC), the cAMP-dependent protein kinase (PKA) [(DeBernardi *et al.* 1996)] and the mitogen-activated-protein kinase Erk-1 [(Sano *et al.* 1995)]. Such methods of detection of intracellular localisation/activity of protein  
25 kinases and phosphatases include immunoprecipitation, Western blotting and immunocytochemical detection.

One aspect of the function of intracellular enzymes which has not been characterised so thoroughly is the redistribution of those enzymes. The importance of subcellular  
30 redistribution of enzymes as a mechanism of enzyme specificity, and of the general importance of the measurement of subcellular redistribution as a tool for identifying novel drug targets and searching for drug candidates which influence those targets, is disclosed in: A METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR RESPONSE, the contents of which

were part of the priority application, and which, as WO9845704 has been published during the priority year, are hereby incorporated herein by reference.

While the redistribution of subcellular components is known to be important, the measurement of this phenomenon in real time has not been widely exploited. This is primarily due to the lack of a suitable technique. There is essentially only one direct technique: the microscopic imaging of cells in which the subcellular component of interest has been labelled in such a way that it can be visualised and recorded by the microscopic imaging system, using for example a video or scientific CCD camera and appropriate software for collecting and storing the images. Novel ways of monitoring specific modulation of intracellular pathways in intact, living cells is assumed to provide new opportunities in drug discovery, functional genomics, toxicology, patient monitoring etc.

15 Recently it was discovered that Green Fluorescent Protein (GFP) expressed in many different cell types, including mammalian cells, became highly fluorescent [(Chalfie *et al.* 1994)]. WO95/07463 describes a cell capable of expressing GFP and a method for detecting a protein of interest in a cell based on introducing into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding a GFP such that the protein produced by the DNA molecule will have the protein of interest fused to the GFP, then culturing the cells in conditions permitting expression of the fused protein and detecting the location of the fluorescence in the cell, thereby localizing the protein of interest in the cell. However, examples of such fused proteins are not provided, and the use of fusion proteins with GFP for detection or quantitation of translocation or redistribution of biologically active polypeptides affecting intracellular processes upon activation, such as proteins involved in signalling pathways, e.g. protein kinases or phosphatases, has not been suggested. WO 95/07463 further describes cells useful for the detection of molecules, such as hormones or heavy metals, in a biological sample, by operatively linking a regulatory element of the gene which is affected by the molecule of interest to a GFP, the presence of the molecules will affect the regulatory element which in turn will affect the expression of the GFP. In this way the gene encoding GFP is used as a reporter gene in a cell which is constructed for monitoring the presence of a specific molecular identity.

Green Fluorescent Protein has been used in an assay for the detection of translocation of the glucocorticoid receptor (GR) [(Carey, KL *et al.* 1996)]. A GR-S65TGFP fusion has been used to study the mechanisms involved in translocation of the glucocorticoid receptor (GR) in response to the agonist dexamethasone from the cytosol, where it is present in the absence of a ligand, through the nuclear pore to the nucleus where it remains after ligand binding. The use of a GR-GFP fusion enables real-time imaging and quantitation of nuclear/cytoplasmic ratios of the fluorescence signal. A similar genetic construct has been used to follow and quantify dexamethasone induced translocation of GR to the nucleus in HeLa cells [(Guiliano, K.A *et al.* 1997)] in a system called Array Scan™ (WO 97/45730) designed for automated drug screening. Recently, several other investigators have demonstrated that tagging a specific protein (or part of a protein) involved in an intracellular signalling pathway with GFP provides a new means to measure and quantify the influence of substances on this pathway. The concept has been shown to work both for cytoplasmic to nuclear translocation of the androgen receptor [(Georget V *et al.* 1997)] and transcription factors such as NF-ATc [(Beals CR *et al.* 1997)] in analogy with what has already been described for GR above. Another relevant example is a  $\beta$ -arrestin – GFP construct that was shown to report on activation of G-protein coupled receptors by translocating from the cytosol to the plasma membrane [(Barak LS *et al.* 1997)]. Finally, it has also been demonstrated that attaching GFP to a smaller part of a protein like the pleckstrin homology domain of phospholipase C  $\delta$  1 [(Stauffer TP *et al.* 1998)] and a cysteine-rich domain of PKC  $\gamma$  [(Oancea E *et al.* 1998)] can be used to report on an influence from a substance by quantifying their redistribution within the cells during activation of the specific signalling pathway to which they belong.

25

Many currently used screening programmes designed to find compounds that affect protein kinase activity are based on measurements of kinase phosphorylation of artificial or natural substrates, receptor binding and/or reporter gene expression. The interest in fluorescence measurements as the basis for future high-throughput drug screening has however increased dramatically over the last few years [(Silverman L *et al.* 1998)]. Of particular interest to the present invention is a scanning laser imager for rapid screening of fluorescence changes in living cells [(Schroeder K & Neagle B 1996)] currently offered commercially by Molecular Devices, Inc. as the FLIPR™.

30

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an important new dimension in the investigation of cellular systems involving redistribution in that the invention provides quantification of the redistribution responses or events caused by an influence, typically contact with a chemical substance or mixture of chemical substances, but also changes in the physical environment, in a massively parallel fashion. The quantification makes it possible to set up meaningful relationships, expressed numerically, or as curves or graphs, between the influences (or the degree of influences) on cellular systems and the redistribution response. This is highly advantageous because, as has been found, the quantification can be achieved in both a fast and reproducible manner, and - what is perhaps even more important - the systems which become quantifiable utilising the method of the invention are systems from which enormous amounts of new information and insight can be derived.

The present screening assays have the distinct advantage over other screening assays, e.g., receptor binding assays, enzymatic assays, and reporter gene assays, in providing a system in which biologically active substances with completely novel modes of action, e.g. inhibition or promotion of redistribution/translocation of a biologically active polypeptide as a way of regulating its action rather than inhibition/activation of enzymatic activity, can be identified in a way that insures very high selectivity to the particular isoform of the biologically active polypeptide and further development of compound selectivity versus other isoforms of the same biologically active polypeptide or other components of the same signalling pathway.

In one of its broadest aspects, the invention relates to an improved method, with higher throughput compared to previous methods, for extracting quantitative information relating to an influence on a cellular response, the method comprising recording variation, caused by the influence on mechanically intact living cells, in spatially distributed light emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, the association resulting in a modulation of the luminescence characteristics of the luminophore, detecting and recording the variation in spatially distributed light from the luminophore as

a change in fluorescence intensity using an instrument designed to measure changes in fluorescence intensity, and processing the recorded variation in the spatially distributed light to provide quantitative information correlating the spatial distribution or change in the spatial distribution to the degree of the influence. In one aspect of the present invention the mechanically intact living cell is permeabilised at some time after the influence has begun but during or before the actual experimental recording. In another aspect, the present invention relates to an improved method for extracting quantitative information relating to an influence on a cellular response, the method comprising recording variation, caused by the influence on permeabilised living cells, in spatially distributed light emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, the association resulting in a modulation of the luminescence characteristics of the luminophore, detecting and recording the spatially distributed light from the luminophore as a change in fluorescence intensity using an instrument designed to measure changes in fluorescence intensity, and processing the recorded variation in the spatially distributed light to provide quantitative information correlating the spatial distribution or change in the spatial distribution to the degree of the influence. In a preferred embodiment of the invention the luminophore, which is present in the cells, is capable of being redistributed by modulation of an intracellular pathway, in a manner which is related to the redistribution of at least one component of the intracellular pathway. In another preferred embodiment of the invention, the luminophore is a fluorophore.

Typically the cell and/or cells are mechanically intact and alive throughout the experiment. In another embodiment of the invention, the cells are fixed at a point in time after the application of the influence at which the response has been predetermined to be significant, and the recording is made at an arbitrary later time. In another embodiment the cell and/or cells are mechanically intact and alive throughout the experiment but are mechanically or chemically disrupted or permeabilised as the initial step of experimental analysis. In another aspect of the invention the cells have their plasma membrane permanently and stably permeabilised before the initiation of the experiment in such a way that the plasma membrane stays permeable during the experiment. This allows the components of intracellular pathways to be contacted by



substances that are not normally permeating the cell plasma membrane such as peptides, proteins and hydrophilic organic compounds..

The mechanically intact or permeabilised living cells could be selected from the group  
5 consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; and vertebrate cells, such as mammalian cells. These cells are incubated at a temperature of 30°C or above, preferably at a temperature of from 32°C to 39°C, more preferably at a temperature of from 35°C to 38°C, and most preferably at a temperature of about 37°C during the time period over which the influence is observed. In one aspect  
10 of the invention the mechanically intact or permeabilised living cell is part of a matrix of identical or non-identical cells. In one embodiment of the invention the cells comprise a group or groups of cells contained within a spatial limitation or spatial limitations. In one embodiment, the cells comprise multiple groups of cells that are qualitatively the same but subjected to different influences. In another embodiment, the cells comprise multiple  
15 groups of cells that are qualitatively different but subjected to the same influence.

In one embodiment of the invention the spatial limitations are domains defined on a substrate on which the cells are present. The spatial limitations may be arranged in one or more arrays on a common carrier. The spatial limitations may be wells in a plate of  
20 microtiter type, such that 96, 384, 864 and 1536 wells are situated on the common carrier. In another embodiment the spatial limitations are wells in a plate of a format different from the microtiter type. In one embodiment of the invention the domains are established by the presence of the cells on the substrate in a pattern that defines the domains. In another aspect of the invention, the domains are instead established by the  
25 spatial pattern or array of the influence or influences as it/they are applied to or contacted by the cells. This aspect is thoroughly disclosed in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference. Briefly, in this aspect of the  
30 invention the mechanically intact or permeabilised living cells are part of a continuous or discontinuous sheet of cells cultured on an optically clear flat surface typically optimised for cell culture. The optically clear and flat surface may be a porous membrane that may allow cellular processes to grow through the membrane pores and may allow directed capillary flow of fluid through the pores.

A cell used in the present invention should contain a nucleic acid construct encoding a fusion polypeptide as defined herein and be capable of expressing the sequence encoded by the construct. The cell is a eukaryotic cell selected from the group consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; vertebrate  
5 cells such as mammalian cells. The preferred cells are mammalian cells.

In another aspect of the invention the cells could be from an organism carrying in at least one of its component cells a nucleic acid sequence encoding a fusion polypeptide as defined herein and be capable of expressing said nucleic acid sequence. The organism  
10 is selected from the group consisting of unicellular and multicellular organisms, such as a mammal.

The luminophore is the component that allows the redistribution to be visualised and/or recorded by emitting light in a spatial distribution related to the degree of influence. The  
15 term redistribution is intended to cover all aspects of a change in spatial location, such as a translocation of the luminophore or other components. In one embodiment of the invention, the luminophore is capable of being redistributed in a manner that is physiologically relevant to the degree of the influence. In another embodiment, the luminophore is capable of associating with a component that is capable of being  
20 redistributed in a manner that is physiologically relevant to the degree of the influence. In another embodiment, a correlation between the redistribution of the luminophore and the degree of the influence could be determined experimentally. In a preferred aspect of the invention, the luminophore is capable of being redistributed in substantially the same manner as the at least one component of an intracellular pathway. In another  
25 embodiment of the invention, the luminophore is capable of being quenched upon spatial association with a component that is redistributed by modulation of the pathway, the quenching being measured as a change in the intensity of the luminescence. In another embodiment of the invention, the luminophore is stationary but may have a certain spatial distribution, and interacts with at least one component that is capable of being  
30 redistributed in a manner which is physiologically relevant to the degree of the influence, in such a way that one or more luminescence characteristics of the luminophore is/are modulated as the component moves closer to, or farther from, the luminophore.

The luminophore could be a fluorophore. In a preferred embodiment of the invention, the  
35 luminophore is a polypeptide encoded by and expressed from a nucleotide sequence

harboured in the cells. The luminophore could be a hybrid polypeptide comprising a fusion of at least a portion of each of two polypeptides one of which comprises a luminescent polypeptide and the other one of which comprises a biologically active polypeptide, as defined herein.

5

The luminescent polypeptide could be a GFP as defined herein or could be selected from the group consisting of green fluorescent proteins having the F64L mutation as defined herein such as F64L-GFP, F64L-Y66H-GFP, F64L-S65T-GFP, and EGFP. The GFP could be N- or C-terminally tagged, optionally via a peptide linker, to the biologically  
10 active polypeptide or a part or a subunit thereof. The fluorescent probe could be a component of an intracellular signalling pathway. The probe is coded for by a nucleic acid construct.

In one aspect of the invention the pathway of investigation is an intracellular signalling  
15 pathway.

In a preferred embodiment of the invention, the influence could be contact between the group or groups of mechanically intact or permeabilised living cells and a chemical substance, and/or incubation of the group or groups of mechanically intact or  
20 permeabilised living cells with a chemical substance in solution. In one aspect of the invention that is thoroughly described in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference, the chemical substances are attached to an  
25 underlying matrix. In this aspect, the chemical substances may also be produced and secreted from, or attached to the plasma membrane surfaces of, a sheet of genetically engineered cells. In this aspect of the invention the chemical substances may also have been separated two-dimensionally in a non-denaturing gel using electrophoresis and the gel is directly put in close proximity or direct contact with the mechanically intact or  
30 permeabilised living cells so that the chemical substances can contact the cells through diffusion or convection.

The influence will modulate the intracellular processes. In one aspect the modulation could be an activation of the intracellular processes. In another aspect the modulation  
35 could be a deactivation of the intracellular processes. In yet another aspect, the

influence could inhibit or promote the redistribution without directly affecting the metabolic activity of the component of the intracellular processes.

In one embodiment the invention is used to establish a dose-response relationship for one or many chemical substances. In one embodiment the invention is used as a basis for a screening program, where the effect of unknown influences such as a compound library, can be compared to influence of known reference compounds under standardised conditions.

10 In addition to the intensity, there are several parameters of fluorescence or luminescence that can be modulated by the effect of the influence on the underlying cellular phenomena, and can therefore be used in the invention. Some examples are resonance energy transfer, fluorescence lifetime, polarisation, and wavelength shift. Each of these methods requires a particular kind of filter in the emission light path to select the  
15 component of the light desired and reject other components. The recording of property of light could be in the form of an ordered array of values such as a CCD array or a vacuum tube device such as a vidicon. In addition, the translational mobility, or freedom of movement, of the luminophore attached to the protein of interest can be an important property affected by the influence on the underlying cellular phenomena, and can  
20 therefore be used in the invention.

In one embodiment of the invention, the spatially distributed light emitted by a luminophore is detected by a change in the resonance energy transfer between the luminophore and another luminescent entity capable of delivering energy to the  
25 luminophore, each of which has been selected or engineered to become part of, bound to or associated with particular components of the intracellular pathway. In this embodiment, either the luminophore or the luminescent entity capable of delivering energy to the luminophore undergoes redistribution in response to an influence. The resonance energy transfer would be measured as a change in the intensity of emission  
30 from the luminophore, preferably sensed by a single channel photodetector that responds only to the average intensity of the luminophore in a non-spatially resolved fashion.

In one embodiment of the invention, the spatially distributed light emitted by a  
35 luminophore includes the case of uniform spatial distribution of the light.

In one aspect of the invention, the luminophore is a fluorophore which redistributes through a non-homogenous excitation light field, resulting in a change in the intensity of the light emitted from the luminophore as a result of the change in the amount of  
5 excitation light intensity at different points in the field.

In one embodiment of the invention, the recording of the spatially distributed light could be made at a single point in time after the application of the influence. In another embodiment, the recording could be made at two points in time, one point being before,  
10 and the other point being after the application of the influence. The result or variation is determined from the change in fluorescence compared to the fluorescence measured prior to the influence or modulation. In another embodiment of the invention, the recording could be performed at a series of points in time, in which the application of the influence occurs at some time after the first time point in the series of recordings, the  
15 recording being performed, e.g., with a predetermined time spacing of from 0.1 seconds to 1 hour, preferably from 1 to 60 seconds, more preferably from 1 to 30 seconds, in particular from 1 to 10 seconds, over a time span of from 1 second to 12 hours, such as from 10 seconds to 12 hours, e.g., from 10 seconds to one hour, such as from 60 seconds to 30 minutes or 20 minutes. The result or variation is determined from the  
20 change in fluorescence over time. The result or variation could also be determined as a change in the spatial distribution of the fluorescence over time.

In one embodiment the recording comprises a time series of total luminescence of the cells of one or several of the spatial limitations. In one embodiment the signal from all of  
25 the spatial limitations, one at a time, is measured by a recording being made in the individual spatial limitations by means of an apparatus to sequentially position each one of the limitations in the field of view of the detector and repeating the positioning and measurement process until all of the spatial limitations have been measured. The detector may be a photomultiplier tube. In a preferred embodiment of the present  
30 invention more than one spatial limitation is measured simultaneously. This may be done by means of a one- or two-dimensional array detector, whereby the multiple spatial limitations are imaged onto the array detector such that discrete subsets of the detecting units (pixels) in the array detector measure the signal from one and only one of the multiple spatial limitations, the signal from any one spatial limitation being the combined  
35 signal from those pixels that receive the image from one of the spatial limitations. This

array detector may be a linear diode array, a video camera (according to any present or future standards and definitions of image acquisition and transmission) or a charge transfer device such as a charge-coupled device (CCD). In one embodiment the recording of signal requires illumination of the multiple spatial limitations to excite the luminophores so that they emit light. In one embodiment all of the spatial limitations are simultaneously illuminated during the measurement. In another embodiment the spatial limitations are singly illuminated only during the time in which they are being measured. In a preferred embodiment the illumination is provided by a laser that is scanned in a raster fashion over some or all of the spatial limitations being measured. The scanning may take place at a rate that is substantially faster than the measurement process such that the illumination appears to the measurement process to be continuous in time and spatially uniform over the region being measured.

The recording of spatially distributed luminescence emitted from the luminophore is performed by an apparatus for measuring the distribution of fluorescence in the cells, and thereby any change in the distribution of fluorescence in the cells, which includes at a minimum the following component parts: (a) a light source, (b) a method for selecting the wavelength(s) of light from the source which will excite the luminescence of the luminophore, (c) a device which can rapidly block or pass the excitation light into the rest of the system, (d) a series of optical elements for conveying the excitation light to the specimen, collecting the emitted fluorescence in a spatially resolved fashion, and forming an image from this fluorescence emission (or another type of intensity map relevant to the method of detection and measurement), (e) a bench or stand which holds the container of the cells being measured in a predetermined geometry with respect to the series of optical elements, (f) a detector to record the spatially resolved fluorescence in the form of an image, (g) a computer or electronic system and associated software to acquire and store the recorded images, and to compute the degree of redistribution from the recorded images.

In a preferred embodiment of the invention the apparatus system is automated. In one embodiment the components in d and e mentioned above comprise a fluorescence microscope. In one embodiment the component in f mentioned above is a CCD camera. In one embodiment the component in f mentioned above is an array of photomultiplier tubes/devices.

In one embodiment the image is formed and recorded by an optical scanning system.

In one embodiment the optical scanning system is used to illuminate the bottom of a plate of microtiter type so that a time-resolved recording of changes in luminescence or  
5 fluorescence can be made from all spatial limitations simultaneously.

In a preferred embodiment the actual luminescence or fluorescence measurements are made in a FLIPR™ instrument, commercially available from Molecular Devices, Inc.

10 In one embodiment of the invention the actual fluorescence measurements are made in a standard type of fluorometer for plates of microtiter type (fluorescence plate reader).

In one embodiment a liquid addition system is used to add a known or unknown compound to any or all of the cells in the cell holder at a time determined in advance.

15 Preferably, the liquid addition system is under the control of the computer or electronic system. Such an automated system can be used for a screening program due to its ability to generate results from a larger number of test compounds than a human operator could generate using the apparatus in a manual fashion.

20 The methods whereby the detector layer of cells are physically contacted by the compounds can also be of another conceptual type where the compounds are delivered to the cells through a porous membrane by convection/diffusion or by directly contacting compounds attached to an inorganic or organic support (such as glass, plastic or the plasma membrane of intact living cells) with the cells. These methods are thoroughly  
25 described in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference, but are also outlined in the following paragraphs.

30 In one aspect of the present invention where the detector layer of cells is a continuous or discontinuous sheet of cells without any separation into test units or wells. The compounds are printed onto a nonabsorbent sheet of porous material as a solution in solvent and allowed to dry. This printed sheet of compounds then defines the test pattern for the experiment as it is brought down in close proximity to or in direct contact with the  
35 underlying detector layer of cells. The compounds, now dissolved by the fluid layer on

the cells, is brought in contact with the cells through the pores of the membrane by convection. The porous membrane onto which the compounds are printed is optically clear and preferably composed as stated in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference. In another embodiment of this aspect of the present invention the detector layer of cells is a continuous or discontinuous sheet of cells, without any separation into test units or wells, growing on a porous and optically clear membrane preferably of the types mentioned above. The porous membrane may allow the cells to send cellular processes through the pores of the membrane. The compounds are printed onto an optically clear substratum such as glass, plastic or quartz as solutions in solvent and allowed to dry. At the time of the experiment the cell sheet on the membrane, surrounded by a thin film of fluid, is layered on top of the printed compound pattern. The compounds then dissolve and contact the cells via diffusion and convection. The compounds may be made using combinatorial chemistry techniques, and may be peptides. The compounds may be covalently attached to the optically clear substratum or porous membrane. The compounds may also be proteins, polypeptides or peptides secreted by or attached to the plasma membrane of genetically modified cells growing as a continuous or discontinuous sheet on a flat optically clear surface or an optically clear porous membrane.

The recording of the variation or result with respect to light emitted from the luminophore is performed by recording the spatially distributed light as one or more digital images, and the processing of the recorded variation to reduce it to one or more numbers representative of the degree of redistribution comprises a digital image processing procedure or combination of digital image processing procedures. The quantitative information which is indicative of the degree of the cellular response to the influence or the result of the influence on the intracellular pathway is extracted from the recording or recordings according to a predetermined calibration based on responses or results, recorded in the same manner, to known degrees of a relevant specific influence. This calibration procedure is developed according to principles described below (Developing an Image-based Assay Technique). Specific descriptions of the procedures for particular assays are given in the examples.



While the stepwise procedure necessary to reduce the image or images to the value representative of the response caused by the influence is particular to each assay, the individual steps are generally well-known methods of image processing. Some examples of the individual steps are point operations such as subtraction, ratioing, and thresholding, digital filtering methods such as smoothing, sharpening, and edge detection, spatial frequency methods such as Fourier filtering, image cross-correlation and image autocorrelation, object finding and classification (blob analysis), and colour space manipulations for visualisation. In addition to the algorithmic procedures, heuristic methods such as neural networks may also be used. In a preferred embodiment of the invention, a dose-response relationship is established based on quantification of the responses caused by a particular influence, representative of the underlying intracellular signalling process, using the methods described above and in the examples. The dose-response relationship for the particular influence is then compared to the dose-response relationship obtained by performing the same assay in an instrument which allows parallel monitoring of all wells in a microtiter plate such as a FLIPR™ or an ordinary fluorescence plate reader for microtiter plates. If a good correlation between the dose-response relationships obtained from the two different measurement systems is obtained, it can be said that the parallel measurement mode has been validated (see examples 8, 9, 10 and 11). This implies that it can be used as the primary basis for a screening assay with the potential benefit of screening a significantly higher number of substances per unit of time for their influence on the response. For example, if the single experiment performed on the microscope can be run in at least 96 experimental chambers simultaneously the throughput for the person who is running the experiments increases by a factor of 96.

25

Imaging plate readers integrate the signal from each well into a single value per time point. Thus the data resulting from a single "run" of the instrument is a set of time series of single values, one for each well, with the injection of the test compound taking place at a known point in the time series. The primary advantage of this type of instrumentation is that it greatly increases the number of samples that can be processed in a given amount of time (the throughput). This is of great advantage when using the assay in a screening program for new pharmaceutical lead compounds.

The first step in the data analysis is to normalise the results from each well so that they can be compared with each other or with previously analysed known compounds. This

always begins with correcting the signal by subtracting the instrument bias from all data points on a well-by-well basis. From this point, either of two techniques can be followed depending on the design of the assay:

Procedure 1: The average of the signal prior to the addition of the test compound is

5 subtracted from all data points on a well-by-well basis.

Procedure 2: The data are corrected for any known background by subtracting the background value from all data points on a well-by-well basis. The resulting background-corrected data are normalised by dividing each data set by the average of the data values prior to the injection of the test compound on a well-by-well basis.

10

The corrected or normalised time series data sets are then further reduced by a technique that converts the time series to a single value. There are at least three such approaches:

For transient responses, the maximum deviation from the baseline is determined. This is  
15 also known as the "peak height" technique.

Alternatively, the signal is integrated over time between pre-defined limits. If the data were treated according to Procedure 2 above, then the offset is subtracted such that the integral of a non-response is zero within the limit of measurement error. This is also  
20 known as the "peak area" technique. If the response is a cumulative one, e.g., an exponential change to a new level, the result is taken as the either the difference or the ratio between the signal after a predetermined time and the signal prior to the addition of the test compound.

25 All of the above procedures reduce the data for a given well to one or more single values. For screening purposes, these values will be searched for those that are greater than a certain statistically determined cut-off value. For characterisation, the values represent a quantitative response, and are further treated in sets by techniques such as dose-response curve fitting.

30

In another embodiment of the invention, the measurement of redistribution is accomplished indirectly by taking advantage of the fact that in order for redistribution to occur, the probe will experience some change in its freedom, or restriction, of movement within the intracellular milieu. The degree of translocation will correlate with the amount  
35 of freely mobile luminophore in the cytoplasm. At a point in time after the test compound

has begun to have any influence it may have, the amount or fraction of restricted luminophore can be measured by disrupting or permeabilising the plasma membrane of the cells and allowing the freely mobile luminophore to diffuse away. If the detection volume of the detector is limited to the region immediately surrounding the cells, and the overall volume into which the freely mobile luminophore can diffuse is much larger, then the freely mobile luminophore essentially disappears from the detector's view and its signal is not recorded.

In one aspect of the invention, the above mentioned measurement of redistribution is made on cells with permanently permeabilised plasma membranes immersed in a solution mimicking the cytoplasmic environment. In this way the influence of compounds that can normally not enter the cytoplasm of cells can be tested.

The nucleic acid constructs used in the present invention encode in their nucleic acid sequences fusion polypeptides comprising a biologically active polypeptide that is a component of an intracellular signalling pathway, or a part thereof, and a GFP, preferably an F64L mutant of GFP, N- or C-terminally fused, optionally via a peptide linker, to the biologically active polypeptide or part thereof. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein kinase or a phosphatase. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a transcription factor or a part thereof which changes cellular localisation upon activation.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein, or a part thereof, which is associated with the cytoskeletal network and which changes cellular localisation upon activation. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein kinase or a part thereof which changes cellular localisation upon activation. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a serine/threonine protein kinase or a part thereof capable of changing intracellular localisation upon activation. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a tyrosine protein kinase or a part thereof capable of changing intracellular localisation upon activation. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a phospholipid-

dependent serine/threonine protein kinase or a part thereof capable of changing intracellular localisation upon activation.

In a specific embodiment the constructs listed in table 1 are used in a method for  
 5 extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed fluorescence emitted from the fluorophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or being modulated by a component which is capable of  
 10 being redistributed in a manner which is related to the degree of the influence, as a change fluorescence intensity preferably measured by an instrument designed for the measurement of changes in fluorescence intensity.

**Table 1** The fusion constructs of the invention by the names used herein as well as by  
 15 reference to relevant SEQ ID NOs of sequences of DNA encoding the construct and full amino acid sequences.

Fusion construct	DNA sequence SEQ ID NO:	Protein Sequence SEQ ID NO:
PKAcat - F64LS65TGFP	1	2
PKC $\alpha$ - F64L-S65TGFP	3	4
EGFP - Erk1	5	6
EGFP - SMAD2	7	8
SMAD2 - EGFP	9	10
EGFP - VASP	11	12
EGFP - NF $\chi$ $\beta$	13	14
NF $\chi$ $\beta$ - EGFP	15	16
EGFP - PKC $\beta$ 1	17	18

As illustrated in examples 8, 9 and 11, the redistribution of PKA, and PKC can readily be detected as a variation in fluorescence intensity, as measured e.g. in the FLIPR<sup>TM</sup> instrument.

20

In one embodiment any new luminophore determined to redistribute in response to an influence in a pattern similar to the pattern observed in the microscope for PKA or PKC (see examples 1, 2, 8 and 11), that is from an aggregated form to a dispersed form or from a dispersed form to an aggregated form of the luminophore as the redistribution  
 25 takes place, can be predicted to be detectable as a variation in light intensity as measured, for example in the FLIPR<sup>TM</sup> instrument.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cAMP-dependent protein kinase or a part thereof capable of changing cellular localisation upon activation. In a preferred embodiment the biologically active polypeptide encoded by the nucleic acid construct is a PKAc-F64L-S65T-GFP fusion. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cGMP-dependent protein kinase or a part thereof capable of changing cellular localisation upon activation.

10 In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a calmodulin-dependent serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a mitogen-activated serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation. In preferred embodiments the biologically active polypeptide encoded by the nucleic acid constructs are an ERK1-F64L-S65T-GFP fusion or an EGFP-ERK1 fusion.

20 In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cyclin-dependent serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein phosphatase or a part thereof capable of changing cellular localisation upon activation.

In one preferred embodiment of the invention the nucleic acid constructs may be DNA constructs.

30

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct. In one embodiment the gene encoding GFP in the nucleic acid construct is derived from *Aequorea victoria*. In a preferred embodiment the gene encoding GFP in the nucleic acid construct is EGFP or a GFP variant selected from F64L-GFP, F64L-

35 Y66H-GFP and F64L-S65T-GFP.

In preferred embodiments of the invention the DNA constructs which can be identified by any of the DNA sequences shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15 and 17 or are variants of these sequences capable of encoding the same fusion polypeptide or a fusion polypeptide which is biologically equivalent thereto, e.g. an isoform, or a splice variant or a homologue from another species.

The present invention describes a method that may be used to establish a screening program for the identification of biologically active substances that directly or indirectly affects intracellular signalling pathways and because of this property are potentially useful as medicaments. Based on measurements in living cells of the redistribution of spatially resolved luminescence from luminophores which undergo a change in distribution upon activation or deactivation of an intracellular signalling pathway the result of the individual measurement of each substance being screened indicates its potential biological activity.

In one embodiment of the invention the screening program is used for the identification of a biologically toxic substance as defined herein that exerts its toxic effect by interfering with an intracellular signalling pathway. Based on measurements in living cells of the redistribution of spatially resolved luminescence from luminophores which undergo a change in distribution upon activation or deactivation of an intracellular signalling pathway the result of the individual measurement of each substance being screened indicates its potential biologically toxic activity. In one embodiment of a screening program a compound that modulates a component of an intracellular pathway as defined herein, can be found and the therapeutic amount of the compound estimated by a method according to the method of the invention. In a preferred embodiment the present invention leads to the discovery of a new way of treating a condition or disease related to the intracellular function of a biologically active polypeptide comprising administration to a patient suffering from said condition or disease of an effective amount of a compound which has been discovered by any method according to the invention. In another preferred embodiment of the invention a method is established for identification of a new drug target or several new drug targets among the group of biologically active polypeptides which are components of intracellular signalling pathways.

- In another embodiment of the invention an individual treatment regimen is established for the selective treatment of a selected patient suffering from an ailment where the available medicaments used for treatment of the ailment are tested on a relevant primary cell or cells obtained from said patient from one or several tissues, using a method
- 5 comprising transfecting the cell or cells with at least one DNA sequence encoding a fluorescent probe according to the invention, transferring the transfected cell or cells back the said patient, or culturing the cell or cells under conditions permitting the expression of said probes and exposing it to an array of the available medicaments, then comparing changes in fluorescence patterns or redistribution patterns of the fluorescent
- 10 probes in the intact living cells to detect the cellular response to the specific medicaments (obtaining a cellular action profile), then selecting one or more medicament or medicaments based on the desired activity and acceptable level of side effects and administering an effective amount of these medicaments to the selected patient.
- 15 The present invention describes a method that may be used to establish a screening program for back-tracking signal transduction pathways as defined herein. In one embodiment the screening program is used to establish more precisely at which level one or several compounds affect a specific signal transduction pathway by successively or in parallel testing the influence of the compound or compounds on the redistribution of
- 20 spatially resolved luminescence from several of the luminophores which undergo a change in distribution upon activation or deactivation of the intracellular signalling pathway under study.

In general, a probe, i.e. a "GeneX"-GFP fusion or a GFP-"GeneX" fusion, is constructed

25 using PCR with "GeneX"-specific primers followed by a cloning step to fuse "GeneX" in frame with GFP. The fusion may contain a short vector derived sequence between "GeneX" and GFP (e.g. part of a multiple cloning site region in the plasmid) resulting in a peptide linker between "GeneX" and GFP in the resulting fusion protein.

- 30 Some of the steps involved in the development of a probe include the following:  
Identify the sequence of the gene. This is most readily done by searching a depository of genetic information, e.g. the GenBank Sequence Database, which is widely available and routinely used by molecular biologists. In the specific examples below the GenBank Accession number of the gene in question is provided.

Design the gene-specific primers. Inspection of the sequence of the gene allows design of gene-specific primers to be used in a PCR reaction. Typically, the top-strand primer encompasses the ATG start codon of the gene and the following ca. 20 nucleotides, while the bottom-strand primer encompasses the stop codon and the ca. 20 preceding 5 nucleotides, if the gene is to be fused behind GFP, i.e. a GFP-"GeneX" fusion. If the gene is to be fused in front of GFP, i.e. a "GeneX"-GFP fusion, a stop codon must be avoided. Optionally, the full-length sequence of GeneX may not be used in the fusion, but merely the part that localizes and redistributes like GeneX in response to a signal. In addition to gene-specific sequences, the primers contain at least one recognition 10 sequence for a restriction enzyme, to allow subsequent cloning of the PCR product. The sites are chosen so that they are unique in the PCR product and compatible with sites in the cloning vector. Furthermore, it may be necessary to include an exact number of nucleotides between the restriction enzyme site and the gene-specific sequence in order to establish the correct reading frame of the fusion gene and/or a translation initiation 15 consensus sequence. Lastly, the primers always contain a few nucleotides in front of the restriction enzyme site to allow efficient digestion with the enzyme.

Identify a source of the gene to be amplified. In order for a PCR reaction to produce a product with gene-specific primers, the gene-sequence must initially be present in the 20 reaction, e.g. in the form of cDNA. Information in GenBank or the scientific literature will usually indicate in which tissue(s) the gene is expressed, and cDNA libraries from a great variety of tissues or cell types from various species are commercially available, e.g. from Clontech (Palo Alto), Stratagene (La Jolla) and Invitrogen (San Diego). Many genes are also available in cloned form from The American Type Tissue Collection (Virginia). 25

Optimise the PCR reaction. Several factors are known to influence the efficiency and specificity of a PCR reaction, including the annealing temperature of the primers, the concentration of ions, notably  $Mg^{2+}$  and  $K^+$ , present in the reaction, as well as pH of the reaction. If the result of a PCR reaction is deemed unsatisfactory, it might be because 30 the parameters mentioned above are not optimal. Various annealing temperatures should be tested, e.g. in a PCR machine with a built-in temperature gradient, available from e.g. Stratagene (La Jolla), and/or various buffer compositions should be tried, e.g. the OptiPrime buffer system from Stratagene (La Jolla).

Clone the PCR product. The vector into which the amplified gene product will be cloned 35 and fused with GFP will already have been taken into consideration when the primers



were designed. When choosing a vector, one should at least consider in which cell types the probe subsequently will be expressed, so that the promoter controlling expression of the probe is compatible with the cells. Most expression vectors also contain one or more selective markers, e.g. conferring resistance to a drug, which is a useful feature when  
5 one wants to make stable transfectants. The selective marker should also be compatible with the cells to be used.

The actual cloning of the PCR product should present no difficulty as it typically will be a one-step cloning of a fragment digested with two different restriction enzymes into a  
10 vector digested with the same two enzymes. If the cloning proves to be problematic, it may be because the restriction enzymes did not work well with the PCR fragment. In this case one could add longer extensions to the end of the primers to overcome a possible difficulty of digestion close to a fragment end, or one could introduce an intermediate cloning step not based on restriction enzyme digestion. Several companies offer systems  
15 for this approach, e.g. Invitrogen (San Diego) and Clontech (Palo Alto).

Once the gene has been cloned and, in the process, fused with the GFP gene, the resulting product, usually a plasmid, should be carefully checked to make sure it is as expected. The most exact test would be to obtain the nucleotide sequence of the fusion-  
20 gene.

Once a DNA construct for a probe has been generated, its functionality and usefulness may be evaluated by transfecting it into cells capable of expressing the probe. The fluorescence of the cell is inspected soon after, typically the next day. At this point, two  
25 features of cellular fluorescence are noted: the intensity and the sub-cellular localisation.

The intensity should usually be at least as strong as that of unfused GFP in the cells. If it is not, the sequence or quality of the probe-DNA might be faulty, and should be carefully checked.  
30

The sub-cellular localisation is an indication of whether the probe is likely to perform well. If it localises as expected for the gene in question, e.g. is excluded from the nucleus, it can immediately go on to a functional test. If the probe is not localised soon after the transfection procedure, it may be because of overexpression at this point in time, as the  
35 cell typically will have taken up very many copies of the plasmid, and localisation will

occur in time, e.g. within a few weeks, as plasmid copy number and expression level decreases. If localisation does not occur after prolonged time, it may be because the fusion to GFP has destroyed a localisation function, e.g. masked a protein sequence essential for interaction with its normal cellular anchor-protein. In this case the opposite fusion might work, e.g. if GeneX-GFP does not work, GFP-GeneX might, as two different parts of GeneX will be affected by the proximity to GFP. If this does not work, the proximity of GFP at either end might be a problem, and it could be attempted to increase the distance by incorporating a longer linker between GeneX and GFP in the DNA construct.

10

If there is no prior knowledge of localisation, and no localisation is observed, it may be because the probe should not be localised at this point, because such is the nature of the protein fused to GFP. It should then be subjected to a functional test.

15 In a functional test, the cells expressing the probe are treated with at least one compound known to perturb, usually by activating, the signalling pathway on which the probe is expected to report by redistributing itself within the cell. If the redistribution is as expected, e.g. if prior knowledge tell that it should translocate from location X to location Y, it has passed the first critical test. In this case it can go on to further characterisation and quantification of the response.

20 If it does not perform as expected, it may be because the cell lacks at least one component of the signalling pathway, e.g. a cell surface receptor, or there is species incompatibility, e.g. if the probe is modelled on sequence information of a human gene product, and the cell is of hamster origin. In both instances one should identify other cell types for the testing process where these potential problems would not apply.

25 If there is no prior knowledge about the pattern of redistribution, the analysis of the redistribution will have to be done in greater depth to identify what the essential and indicative features are, and when this is clear, it can go on to further characterisation and quantification of the response. If no feature of redistribution can be identified, the problem might be as mentioned above, and the probe should be retested under more optimal cellular conditions. If the probe does not perform under optimal cellular conditions, then it's back to the drawing board.

35

The process of developing an image-based redistribution assay begins with either the unplanned experimental observation that a redistribution phenomenon can be visualised, or the design of a probe specifically to follow a redistribution phenomenon already known to occur. In either event, the first and best exploratory technique is for a trained scientist  
5 or technician to observe the phenomenon. Even with the rapid advances in computing technology, the human eye-brain combination is still the most powerful pattern recognition system known, and requires no advance knowledge of the system in order to detect potentially interesting and useful patterns in raw data. This is especially if those data are presented in the form of images, which are the natural "data type" for human  
10 visual processing. Because human visual processing operates most effectively in a relatively narrow frequency range, i.e., we cannot see either very fast or very slow changes in our visual field, it may be necessary to record the data and play it back with either time dilation or time compression.

15 Some luminescence phenomena cannot be seen directly by the human eye. Examples include polarisation and fluorescence lifetime. However, with suitable filters or detectors, these signals can be recorded as images or sequences of images and displayed to the human in the fashion just described. In this way, patterns can be detected and the same methods can be applied.

20

Once the redistribution has been determined to be a reproducible phenomenon, one or more data sets are generated for the purpose of developing a procedure for extracting the quantitative information from the data. In parallel, the biological and optical conditions are determined which will give the best quality raw data for the assay. This  
25 can become an iterative process; it may be necessary to develop a quantitative procedure in order to assess the effect on the assay of manipulating the assay conditions.

The data sets are examined by a person or persons with knowledge of the biological  
30 phenomenon and skill in the application of image processing techniques. The goal of this exercise is to determine or at least propose a method that will reduce the image or sequence of images constituting the record of a "response" to a value corresponding to the degree of the response. Using either interactive image processing software or an image processing toolbox and a programming language, the method is encoded as a  
35 procedure or algorithm that takes the image or images as input and generates the

degree of response (in any units) as its output. Some of the criteria for evaluating the validity of a particular procedure are:

Does the degree of the response vary in a biologically significant fashion, i.e., does it  
5 show the known or putative dependence on the concentration of the stimulating agent or condition?

Is the degree of response reproducible, i.e., does the same concentration or level of  
stimulating agent or condition give the same response with an acceptable variance? Is  
10 the dynamic range of the response sufficient for the purpose of the assay? If not, can a change in the procedure or one of its parameters improve the dynamic range? Does the procedure exhibit any clear "pathologies", i.e., does it give ridiculous values for the response if there are commonly occurring imperfections in the imaging process? Can these pathologies be eliminated, controlled, or accounted for? Can the procedure deal  
15 with the normal variation in the number and/or size of cells in an image?

In some cases the method may be obvious; in others, a number of possible procedures may suggest themselves. Even if one method appears clearly superior to others, optimisation of parameters may be required. The various procedures are applied to the  
20 data set and the criteria suggested above are determined, or the single procedure is applied repeatedly with adjustment of the parameter or parameters until the most satisfactory combination of signal, noise, range, etc. are arrived at. This is equivalent to the calibration of any type of single-channel sensor.

25 The number of ways of extracting a single value from an image are extremely large, and thus an intelligent approach must be taken to the initial step of reducing this number to a small, finite number of possible procedures. This is not to say that the procedure arrived at is necessarily the best procedure - but a global search for the best procedure is simply out of the question due to the sheer number of possibilities involved.

30

Image-based assays are no different than other assay techniques in that their usefulness is characterised by parameters such as the specificity for the desired component of the sample, the dynamic range, the variance, the sensitivity, the concentration range over which the assay will work, and other such parameters. While it is not necessary to

characterise each and every one of these before using the assay, they represent the only way to compare one assay with another.

The final step is then to see whether there exists a possibility to increase the throughput of the assay to improve its utility as the basis of a screening program. In order to do this, a dose-response relationship is established based on quantification of the responses caused by a particular influence, representative of the underlying intracellular signalling process, using the methods described above and in the examples. The dose-response relationship for the particular influence is then compared to the dose-response relationship obtained by performing the same assay in an instrument which allows parallel monitoring of all wells in a microtiter plate such as a FLIPR™ or an ordinary imaging or fluorescence plate reader for microtiter plates. If a good correlation between the dose-response relationships obtained from the two different measurement systems is obtained, it can be said that the parallel measurement mode has been validated (see examples 8, 9 and 11). This implies that it can be used as the primary basis for a screening program with the potential benefit of screening a significantly higher number of substances for their influence on the response per unit of time.

In the present specification and claims, the term "an influence" covers any influence to which the cellular response comprises a redistribution. Thus, e.g., heating, cooling, pH, high pressure, low pressure, humidifying, or drying are influences on the cellular response on which the resulting redistribution can be quantified, but as mentioned above, perhaps the most important influences are the influences of contacting or incubating the cells with substances which are known or suspected to exert an influence on the cellular response involving a redistribution contribution. In another embodiment of the invention the influence could be substances from a compound drug library.

In the present context, the term "green fluorescent protein" is intended to indicate a protein which, when expressed by a cell, emits fluorescence upon exposure to light of the correct excitation wavelength (cf. [(Chalfie, M. *et al.* (1994) *Science* 263, 802-805])). In the following, GFP in which one or more amino acids have been substituted, inserted or deleted is most often termed "modified GFP". "GFP" as used herein includes wild-type GFP derived from the jelly fish *Aequorea victoria* and modifications of GFP, such as the blue fluorescent variant of GFP disclosed by Heim *et al.* (1994). *Proc.Natl.Acad.Sci.* 91:26, pp 12501-12504, and other modifications that change the spectral properties of the GFP

fluorescence, or modifications that exhibit increased fluorescence when expressed in cells at a temperature above about 30°C described in PCT/DK96/00051, published as WO 97/11094 on 27 March 1997 and hereby incorporated by reference, and which comprises a fluorescent protein derived from *Aequorea* Green Fluorescent Protein (GFP) or any functional analogue thereof, wherein the amino acid in position 1 upstream from the chromophore has been mutated to provide an increase of fluorescence intensity when the fluorescent protein of the invention is expressed in cells. Preferred GFP variants are F64L-GFP, F64L-Y66H-GFP and F64L-S65T-GFP. An especially preferred variant of GFP for use in all the aspects of this invention is EGFP (DNA encoding EGFP which is a F64L-S65T variant with codons optimized for expression in mammalian cells is available from Clontech, Palo Alto, plasmids containing the EGFP DNA sequence, cf. GenBank Acc. Nos. U55762, U55763).

The term "intracellular signalling pathway" and "signal transduction pathway" are intended to indicate the co-ordinated intracellular processes whereby a living cell transduce an external or internal signal into cellular responses. Said signal transduction will involve an enzymatic reaction said enzymes include but are not limited to protein kinases, GTPases, ATPases, protein phosphatases, phospholipases and cyclic nucleotide phosphodiesterases. The cellular responses include but are not limited to gene transcription, secretion, proliferation, mechanical activity, metabolic activity, cell death.

The term "second messenger" is used to indicate a low molecular weight component involved in the early events of intracellular signal transduction pathways.

The term "luminophore" is used to indicate a chemical substance that has the property of emitting light either inherently or upon stimulation with chemical or physical means. This includes but is not limited to fluorescence, bioluminescence, phosphorescence, and chemiluminescence.

The term "mechanically intact living cell" is used to indicate a cell which is considered living according to standard criteria for that particular type of cell such as maintenance of normal membrane potential, energy metabolism, proliferative capability, and has not experienced any physically invasive treatment designed to introduce external substances into the cell such as microinjection.

In the present context, the term "permeabilised living cell" is used to indicate cells where a pore forming agent such as Streptolysin O or *Staphylococcus Aureus*  $\alpha$ -toxin has been applied and thereby incorporated into the plasma membrane in the cells. This creates  
5 proteinaceous pores with a defined pore size in the plasma membranes of the exposed cells. Pores could also be made by electroporation, i.e. exposing the cells to high voltage discharges, a procedure that creates small holes in the plasma membrane by coagulating integral membrane proteins. Treatment with a mild detergent such as saponin may accomplish the same thing. Common to all these treatments are that pores  
10 are formed only in the plasma membrane without affecting the integrity of cytoplasmic structural elements and organelles. The term living in this context means that the permeabilised cells bathed in a solution mimicking the intracellular milieu still have functional organelles, such as actively respiring mitochondria and endoplasmic reticulum that can take up and release calcium ions, and functional structural elements. The  
15 benefit of this method is that substances that normally can not traverse the plasma membrane, but most likely exert their influence intracellularly, can be introduced and their influence studied without cumbersome microinjection of the substances into single cells. Using this method the response to an influence can be recorded from many cells simultaneously.

20

In the present context, the term "permeabilisation" is intended to indicate the selective disruption of the plasma membrane barrier so that soluble substances freely mobile in the cytosol are lost from the cells. The permeabilisation can be achieved as described above under "permeabilised living cells" or by using other chemical detergents such as  
25 Triton X-100 or digitonin in carefully titrated amounts.

The term "physiologically relevant", when applied to an experimentally determined redistribution of an intracellular component, as measured by a change in the luminescence properties or distribution, is used to indicate that said redistribution can be  
30 explained in terms of the underlying biological phenomenon which gives rise to the redistribution.

The terms "image processing" and "image analysis" are used to describe a large family of digital data analysis techniques or combination of such techniques which reduce  
35 ordered arrays of numbers (images) to quantitative information describing those ordered

arrays of numbers. When said ordered arrays of numbers represent measured values from a physical process, the quantitative information derived is therefore a measure of the physical process.

- 5 The term "fluorescent probe" is used to indicate a fluorescent fusion polypeptide comprising a GFP or any functional part thereof which is N- or C-terminally fused to a biologically active polypeptide as defined herein, optionally via a peptide linker consisting of one or more amino acid residues, where the size of the linker peptide in itself is not critical as long as the desired functionality of the fluorescent probe is maintained. A  
10 fluorescent probe according to the invention is expressed in a cell and basically mimics the physiological behaviour of the biologically active polypeptide moiety of the fusion polypeptide.

The term "mammalian cell" is intended to indicate any living cell of mammalian origin.

- 15 The cell may be an established cell line, many of which are available from The American Type Culture Collection (ATCC, Virginia, USA) or a primary cell with a limited life span derived from a mammalian tissue, including tissues derived from a transgenic animal, or a newly established immortal cell line derived from a mammalian tissue including transgenic tissues, or a hybrid cell or cell line derived by fusing different cell types of  
20 mammalian origin e.g. hybridoma cell lines. The cells may optionally express one or more non-native gene products, e.g. receptors, enzymes, enzyme substrates, prior to or in addition to the fluorescent probe. Preferred cell lines include but are not limited to those of fibroblast origin, e.g. BHK, CHO, BALB, or of endothelial origin, e.g. HUVEC, BAE (bovine artery endothelial), CPAE (cow pulmonary artery endothelial), HLMVEC  
25 (human lung microvascular endothelial cells) or of pancreatic origin, e.g. RIN, INS-1, MIN6, bTC3, aTC6, bTC6, HIT, or of hematopoietic origin, e.g. primary isolated human monocytes, macrophages, neutrophils, basophils, eosinophils and lymphocyte populations, AML-193, HL-60, RBL-1, adipocyte origin, e.g. 3T3-L1, neuronal/neuroendocrine origin, e.g. AtT20, PC12, GH3, muscle origin, e.g. SKMC, A10,  
30 C2C12, renal origin, e.g. HEK 293, LLC-PK1.

- The term "hybrid polypeptide" is intended to indicate a polypeptide which is a fusion of at least a portion of each of two proteins, in this case at least a portion of the green fluorescent protein, and at least a portion of a catalytic and/or regulatory domain of a  
35 protein kinase. Furthermore a hybrid polypeptide is intended to indicate a fusion



polypeptide comprising a GFP or at least a portion of the green fluorescent protein that contains a functional fluorophore, and at least a portion of a biologically active polypeptide. Thus, GFP may be N- or C-terminally tagged to a biologically active polypeptide, optionally via a linker portion or linker peptide consisting of a sequence of  
5 one or more amino acids. The hybrid polypeptide or fusion polypeptide may act as a fluorescent probe in intact living cells carrying a DNA sequence encoding the hybrid polypeptide under conditions permitting expression of said hybrid polypeptide.

The term "kinase" is intended to indicate an enzyme that is capable of phosphorylating a  
10 cellular component.

The term "protein kinase" is intended to indicate an enzyme that is capable of phosphorylating serine and/or threonine and/or tyrosine in peptides and/or proteins.

15 The term "phosphatase" is intended to indicate an enzyme that is capable of dephosphorylating phosphoserine and/or phosphothreonine and/or phosphotyrosine in peptides and/or proteins.

The term "cyclic nucleotide phosphodiesterase" is intended to indicate an enzyme that is  
20 capable of inactivating the second messengers cAMP and cGMP by hydrolysis of their 3'-ester bond.

In the present context, the term "biologically active polypeptide" is intended to indicate a polypeptide affecting intracellular processes upon activation, such as an enzyme which  
25 is active in intracellular processes or a portion thereof comprising a desired amino acid sequence which has a biological function or exerts a biological effect in a cellular system. In the polypeptide one or several amino acids may have been deleted, inserted or replaced to alter its biological function, e.g. by rendering a catalytic site inactive.

Preferably, the biologically active polypeptide is selected from the group consisting of  
30 proteins taking part in an intracellular signalling pathway, such as enzymes involved in the intracellular phosphorylation and dephosphorylation processes including kinases, protein kinases and phosphorylases as defined herein, but also proteins making up the cytoskeleton play important roles in intracellular signal transduction and are therefore included in the meaning of "biologically active polypeptide" herein. More preferably, the  
35 biologically active polypeptide is a protein which according to its state as activated or

non-activated changes localisation within the cell, preferably as an intermediary component in a signal transduction pathway. Included in this preferred group of biologically active polypeptides are cAMP dependent protein kinase A.

- 5 The term "a substance having biological activity" is intended to indicate any sample that has a biological function or exerts a biological effect in a cellular system. The sample may be a sample of a biological material such as a sample of a body fluid including blood, plasma, saliva, milk, urine, or a microbial or plant extract, an environmental sample containing pollutants including heavy metals or toxins, or it may be a sample  
10 containing a compound or mixture of compounds prepared by organic synthesis or genetic techniques.

- The phrase "any change in fluorescence" means any change in absorption properties, such as wavelength and intensity, or any change in spectral properties of the emitted  
15 light, such as a change of wavelength, fluorescence lifetime, intensity or polarisation, or any change in the intracellular localisation of the fluorophore. It may thus be localised to a specific cellular component (e.g. organelle, membrane, cytoskeleton, molecular structure) or it may be evenly distributed throughout the cell or parts of the cell.

- 20 The term "organism" as used herein indicates any unicellular or multicellular organism preferably originating from the animal kingdom including protozoans, but also organisms that are members of the plant kingdoms, such as algae, fungi, bryophytes, and vascular plants are included in this definition.
- 25 The term "nucleic acid" is intended to indicate any type of poly- or oligonucleic acid sequence, such as a DNA sequence, a cDNA sequence, or an RNA sequence.

- The term "biologically equivalent" as it relates to proteins is intended to mean that a first protein is equivalent to a second protein if the cellular functions of the two proteins may  
30 substitute for each other, e.g. if the two proteins are closely related isoforms encoded by different genes, if they are splicing variants, or allelic variants derived from the same gene, if they perform identical cellular functions in different cell types, or in different species. The term "biologically equivalent" as it relates to DNA is intended to mean that a first DNA sequence encoding a polypeptide is equivalent to a second DNA sequence

encoding a polypeptide if the functional proteins encoded by the two genes are biologically equivalent.

The term "higher throughput" is intended to mean an increased number of experiments  
5 per time unit per person performing the actual experiments.

The term "high throughput screening assay" as used herein is intended to mean the process of performing a screening assay with at least 100 individual experiments where compounds are tested for their influence on the redistribution of a luminophore in one  
10 working day for one person skilled in the art. In a preferred embodiment the high throughput screening assay involves at least 500 individual experiments such as 750, 1000, 2000, 5000, or even 10.000 individual experiments in one working day for a person skilled in the art.

15 The phrase "back-tracking of a signal transduction pathway" is intended to indicate a process for defining more precisely at what level a signal transduction pathway is affected, either by the influence of chemical compounds or a disease state in an organism. Consider a specific signal transduction pathway represented by the bioactive polypeptides A - B - C - D, with signal transduction from A towards D. When investigating  
20 all components of this signal transduction pathway compounds or disease states that influence the activity or redistribution of only D can be considered to act on C or downstream of C whereas compounds or disease states that influence the activity or redistribution of C and D, but not of A and B can be considered to act downstream of B.

25 The term "fixed cells" is used to mean cells treated with a cytological fixative such as glutaraldehyde or formaldehyde, treatments that serve to chemically cross-link and stabilise soluble and insoluble proteins within the structure of the cell. Once in this state, such proteins cannot be lost from the structure of the now-dead cell.

30 In the present context a "screening assay" is intended to mean any measurement protocol, including materials, cells, instruments, chemicals, reagents, detection units, calibration and quantification procedures used to measure a response from mechanically intact or permeabilised living cells relevant to influences on an intracellular pathway.

The term "dose-response relationship" and "screening programme" is in the present context intended to mean a clear correlation between the quantified response of cells in a screening assay to application of an influence, such as a compound, and the concentration of the applied influence. The response to the influence may be both an up-  
5 regulation and a down-regulation of the quantified parameter used in the screening assay.

In the present context, the term "physiology" is intended to mean the normal function of biological and biochemical processes inside cells, between cells and in the whole  
10 organism or animal.

## BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1. CHO cells expressing the PKAc-F64L-S65T-GFP hybrid protein have been treated in HAM's F12 medium with 50  $\mu$ M forskolin at 37°C. The images of the GFP  
15 fluorescence in these cells have been taken at different time intervals after treatment, which were: a) 40 seconds b) 60 seconds c) 70 seconds d) 80 seconds. The fluorescence changes from a punctate to a more even distribution within the (non-nuclear) cytoplasm.
- 20 Figure 2. Time-lapse analysis of forskolin induced PKAc-F64L-S65T-GFP redistribution. CHO cells, expressing the PKAc-F64L-S65T-GFP fusion protein were analysed by time-lapse fluorescence microscopy. Fluorescence micrographs were acquired at regular intervals from 2 min before to 8 min after the addition of agonist. The cells were challenged with 1  $\mu$ M forskolin immediately after the upper left image was acquired (t=0).  
25 Frames were collected at the following times: i) 0, ii) 1, iii) 2, iv) 3, v) 4 and vi) 5 minutes. Scale bar 10  $\mu$ m.

- Figure 3. Time-lapse analyses of PKAc-F64L-S65T-GFP redistribution in response to various agonists. The effects of 1  $\mu$ M forskolin (A), 50  $\mu$ M forskolin (B), 1mM dbcAMP  
30 (C) and 100  $\mu$ M IBMX (D) (additions indicated by open arrows) on the localisation of the PKAc-F64L-S65T-GFP fusion protein were analysed by time-lapse fluorescence microscopy of CHO/PKAc-F64L-S65T-GFP cells. The effect of addition of 10  $\mu$ M forskolin (open arrow), followed shortly by repeated washing with buffer (solid arrow), on the localisation of the PKAc-F64L-S65T-GFP fusion protein was analysed in the same

cells (E). In a parallel experiment, the effect of adding 10  $\mu$ M forskolin and 100  $\mu$ M IBMX (open arrow) followed by repeated washing with buffer containing 100  $\mu$ M IBMX (solid arrow) was analysed (F). Removing forskolin caused PKAc-F64L-S65T-GFP fusion protein to return to the cytoplasmic aggregates while this is prevented by the continued presence of IBMX (F). The effect of 100 nM glucagon (Fig 3G, open arrow) on the localisation of the PKAc-F64L-S65T-GFP fusion protein is also shown for BHK/GR, PKAc-F64L-S65T-GFP cells. The effect of 10  $\mu$ M norepinephrine (H), solid arrow, on the localisation of the PKAc-F64L-S65T-GFP fusion protein was analysed similarly, in transiently transfected CHO, PKAc-F64L-S65T-GFP cells, pretreated with 10  $\mu$ M forskolin, open arrow, to increase [cAMP]. N.B. in Fig 3H the x-axis counts the image numbers, with 12 seconds between images. The raw data of each experiment consisted of 60 fluorescence micrographs acquired at regular intervals including several images acquired before the addition of buffer or agonist. The charts (A-G) each show a quantification of the response seen through all the 60 images, performed as described in analysis method 2. The change in total area of the highly fluorescent aggregates, relative to the initial area of fluorescent aggregates is plotted as the ordinate in all graphs in Figure 3, versus time for each experiment. Scale bar 10  $\mu$ m.

Figure 4. Dose-response curve (two experiments) for forskolin-induced redistribution of the PKAc-F64L-S65T-GFP fusion.

Figure 5. Time from initiation of a response to half maximal ( $t_{1/2max}$ ) and maximal ( $t_{max}$ ) PKAc-F64L-S65T-GFP redistribution. The data was extracted from curves such as that shown in "Figure 2." All  $t_{1/2max}$  and  $t_{max}$  values are given as mean $\pm$ SD and are based on a total of 26-30 cells from 2-3 independent experiments for each forskolin concentration. Since the observed redistribution is sustained over time, the  $t_{max}$  values were taken as the earliest time point at which complete redistribution is reached. Note that the values do not relate to the degree of redistribution.

Figure 6. Parallel dose-response analyses of forskolin induced cAMP elevation and PKAc-F64L-S65T-GFP redistribution. The effects of buffer or increasing concentrations of forskolin on the localisation of the PKAc-F64L-S65T-GFP fusion protein in CHO/PKAc-F64L-S65T-GFP cells, grown in a 96 well plate, were analysed as described above. Computing the ratio of the SD's of fluorescence micrographs taken of the same field of cells, prior to and 30 min after the addition of forskolin, gave a reproducible measure of

PKAc-F64L-S65T-GFP redistribution. The graph shows the individual 48 measurements and a trace of their mean  $\pm$  s.e.m at each forskolin concentration. For comparison, the effects of buffer or 8 increasing concentrations of forskolin on  $[cAMP]_i$  was analysed by a scintillation proximity assay of cells grown under the same conditions. The graph shows  
 5 a trace of the mean  $\pm$  s.e.m of 4 experiments expressed in arbitrary units.

Figure 7. BHK cells stably transfected with the human muscarinic (hM1) receptor and the PKC $\alpha$ -F64L-S65T-GFP fusion. Carbachol (100  $\mu$ M added at 1.0 second) induced a transient redistribution of PKC $\alpha$ -F64L-S65T-GFP from the cytoplasm to the plasma  
 10 membrane. Images were taken at the following times: a) 1 second before carbachol addition, b) 8.8 seconds after addition and c) 52.8 seconds after addition.

Figure 8. BHK cells stably transfected with the hM1 receptor and PKC $\alpha$ -F64L-S65T-GFP fusion were treated with carbachol (1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M). In single cells intracellular  
 15  $[Ca^{2+}]$  was monitored simultaneously with the redistribution of PKC $\alpha$ -F64L-S65T-GFP. Dashed line indicates the addition times of carbachol. The top panel shows changes in the intracellular  $Ca^{2+}$  concentration of individual cells with time for each treatment. The middle panel shows changes in the average cytoplasmic GFP fluorescence for individual cells against time for each treatment. The bottom panel shows changes in the  
 20 fluorescence of the periphery of single cells, within regions that specifically include the circumferential edge of a cell as seen in normal projection, the best regions for monitoring changes in the fluorescence intensity of the plasma membrane.

Figure 9. The hERK1-F64L-S65T-GFP fusion expressed in HEK293 cells treated with  
 25 100  $\mu$ M of the MEK1 inhibitor PD98059 in HAM F-12 (without serum) for 30 minutes at 37 °C. The nuclei empty of fluorescence during this treatment. The same cells as in (a) following treatment with 10 % foetal calf serum for 15 minutes at 37 °C.

Time profiles for the redistribution of GFP fluorescence in HEK293 cells following treatment with various concentrations of EGF in Hepes buffer (HAM F-12 replaced with  
 30 Hepes buffer directly before the experiment). Redistribution of fluorescence is expressed as the change in the ratio value between areas in nucleus and cytoplasm of single cells. Each time profile is the mean for the changes seen in six single cells. Bar chart for the end-point measurements, 600 seconds after start of EGF treatments, of fluorescence change (nucleus:cytoplasm) following various concentrations of EGF.

Figure 10. The SMAD2-EGFP fusion expressed in HEK293 cells starved of serum overnight in HAM F-12. HAM F-12 was then replaced with Hepes buffer pH 7.2 immediately before the experiment. Scale bar is 10  $\mu$ m.

HEK 293 cells expressing the SMAD2-EGFP fusion were treated with various

5 concentration of TGF-beta as indicated, and the redistribution of fluorescence monitored against time. The time profile plots represent increases in fluorescence within the nucleus, normalised to starting values in each cell measured. Each trace is the time profile for a single cell nucleus.

A bar chart representing the end-point change in fluorescence within nuclei (after 850  
10 seconds of treatment) for different concentrations of TGF-beta. Each bar is the value for a single nucleus in each treatment.

Figure 11. The VASP-F64L-S65T-GFP fusion in CHO cells stably transfected with the human insulin receptor. The cells were starved for two hours in HAM F-12 without  
15 serum, then treated with 10% foetal calf serum. The image shows the resulting redistribution of fluorescence after 15 minutes of treatment. GFP fluorescence becomes localised in structures identified as focal adhesions along the length of actin stress fibres.

Figure 12. Dose-response relationship for the translocation of PKC $\alpha$ -GFP in BHKhM1  
20 cells stimulated with the muscarinic agonist carbamylcholine using a FLIPR™ to do the actual experiments.

Figure 13. Dose-response relationship for the translocation of PKAc-GFP in CHO/PKAc-  
F64L-S65T-GFP cells stimulated with forskolin using a FLIPR™ to do the actual  
25 experiments.

Figure 14. CHO cells stably expressing the human insulin receptor and mouse cPKA labeled with S65T-GFP were more thoroughly investigated in the FLIPR™ instrument. A forskolin (a substance that increases Adenylate cyclase production of cAMP in the cells)  
30 dose-response was created where six separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC (area under the curve) for 9 min of stimulation.

Conclusion: Redistribution of mouse cPKA - BioST can be detected in the FLIPR™  
despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated  
35 and imaged simultaneously with a spatial resolution that is far from capable of resolving

single cells or subcellular events. The method can be used as a real time measurement of cAMP levels in the cells and as a screening assay to measure effects of ligands to G-protein coupled receptors linked to Gi and Gq type G-proteins.

- 5 Figure 15. Dose-response relationship for the disappearance of fluorescence from permeabilised CHO/PKAc-F64L-S65T-GFP when previously exposed to different doses of forskolin.

Figure 16. CHO cells stably expressing the human insulin receptor and human PKC beta

- 10 1 labeled with EGFP were investigated in the microscope. A dose-response was created where a set of cells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 4 min of stimulation. From the images the following data were extracted:

Whole image : Just analysing the change in intensity in the whole images taking both

- 15 cells and background.

Single cell: 5 separate cells were analysed after background compensation. The analysis was made on the entire cell.

Cytoplasm: The same 5 cells as above were analysed after background compensation. the analysis was made on a small region in the cytoplasm close to the nucleus.

- 20 Conclusion: Redistribution of human PKC beta 1 – EGFP can only be detected if a subregion of each cell is analysed. The event is clearly visible when the image series is viewed as a movie but if the whole image change in fluorescence or the change in fluorescence in entire cells are analysed the redistribution cannot be detected.

- 25 Figure 17. CHO cells stably expressing the human insulin receptor and human PKC beta 1 labeled with EGFP were investigated in the FLIPR™. A dose-response was created where six separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 5 min of stimulation.

Conclusion: Redistribution of human PKC beta 1 – EGFP can be detected in the

- 30 FLIPR™ despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated and imaged by a detector that has a resolution far below that needed to resolve single cells or subcellular structures. This phenomenon can clearly not be predicted from the microscope data in Figure 16.



- Figure 18 CHO cells stably expressing the insulin receptor and a human NFkB – GFP protein hybrid were stimulated with different concentrations of IL-1 for 1 h, then washed with a hypoosmotic buffer (TRIS-base 10mM, MgCl<sub>2</sub> 2mM, PMSF(Phenyl methyl sulfonyl fluoride) 0.5mM, pH 7.4) and placed on the microscope. A sequence of images were
- 5 acquired during the addition of 0.05% Triton X-100 and subsequent gentle mixing after a short incubation period. The treatment causes the cell membranes to rupture leaving the fraction of NFkB-GFP that has translocated to the nucleus behind whereas the cytoplasmic amount of the probe leaves the cells more quickly and immediately becomes infinitely diluted in the surrounding medium (out of focus - this part of the total
- 10 fluorescence from the probe is thereby lost). At a defined time point before and after this treatment a total intensity value for the whole image was extracted. To normalize each experiment, the after value was divided by the before value. meaning that a higher ratio was found in cells where more NFkB had translocated to the nucleus and thereby contributed to the total fluorescence after permeabilisation.
- 15 Conclusion: the present protocol is a good example of the possibility of revealing translocation of a fluorescent probe from the cytosol to the nucleus or translocation from the nucleus to the cytosol.

## EXAMPLES

### **EXAMPLE 1 Construction, testing and implementation of an assay for cAMP based on PKA activation.**

- 5 Useful for monitoring the activity of signalling pathways that lead to altered concentrations of cAMP, e.g. activation of G-protein coupled receptors which couple to G-proteins of the G<sub>s</sub> or G<sub>i</sub> class.

The catalytic subunit of the murine cAMP dependent protein kinase (PKAc) was fused C-terminally to a F64L-S65T derivative of GFP. The resulting fusion (PKAc-F64L-S65T-

- 10 GFP) was used for monitoring *in vivo* the translocation and thereby the activation of PKA.

To construct the PKAc-F64L-S65T-GFP fusion, convenient restriction endonuclease sites were introduced into the cDNAs encoding murine PKAc (Gen Bank Accession number: M12303) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) by

- 15 polymerase chain reaction (PCR). The PCR reactions were performed according to standard protocols with the following primers:

5'PKAc:

TTggACACAAgCTTTggACACCCTCAggATATgggCAACgCCgCCgCCgCCAAg,

3'PKAc:

- 20 gTCATCTTCTCgAgTCTTTCAGgCgCgCCCAAACCTCAgTAAACTCCTTgCCACAC

5'GFP:

TTggACACAAgCTTTggACACggCgCgCCATgAgTAAAggAgAAgAACTTTTC

3'GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT.

25

The PKAc amplification product was then digested with HindIII+Ascl and the F64L-S65T-GFP product with Ascl+XhoI. The two digested PCR products were subsequently ligated with a HindIII+XhoI digested plasmid (pZeoSV® mammalian expression vector, Invitrogen, San Diego, CA, USA). The resulting fusion construct (SEQ ID NO:1 and 2)

- 30 was under control of the SV40 promoter.

Transfection and cell culture conditions:

- Chinese hamster ovary cells (CHO), were transfected with the plasmid containing the PKAc-F64L-S65T-GFP fusion using the calcium phosphate precipitate method in HEPES-buffered saline (Sambrook *et al.*, 1989). Stable transfectants were selected using 1000  $\mu\text{g}$  Zeocin/ml (Invitrogen) in the growth medium (DMEM with 1000 mg glucose/l, 10 % fetal bovine serum (FBS), 100  $\mu\text{g}$  penicillin-streptomycin mixture  $\text{ml}^{-1}$ , 2 mM L-glutamine purchased from Life Technologies Inc., Gaithersburg, MD, USA). Untransfected CHO cells were used as the control. To assess the effect of glucagon on fusion protein translocation, the PKAc-F64L-S65T-GFP fusion was stably expressed in baby hamster kidney cells overexpressing the human glucagon receptor (BHK/GR cells).
- 10 Untransfected BHK/GR cells were used as the control. Expression of GR was maintained with 500  $\mu\text{g}$  G418/ml (*Neo* marker) and PKAc-F64L-S65T-GFP was maintained with 500  $\mu\text{g}$  Zeocin/ml (*Sh ble* marker). CHO cells were also simultaneously co-transfected with vectors containing the PKAc-F64L-S65T-GFP fusion and the human  $\alpha 2\text{a}$  adrenoceptor (hARa2a).
- 15 For fluorescence microscopy, cells were allowed to adhere to Lab-Tek chambered coverglasses (Nalge Nunc Int., Naperville, IL, USA) for at least 24 hours and cultured to about 80% confluence. Prior to experiments, the cells were cultured over night without selection pressure in HAM F-12 medium with glutamax (Life Technologies), 100  $\mu\text{g}$  penicillin-streptomycin mixture  $\text{ml}^{-1}$  and 0.3 % FBS. This medium has low
- 20 autofluorescence enabling fluorescence microscopy of cells straight from the incubator.

Monitoring activity of PKA activity in real time:

- Image acquisition of live cells were gathered using a Zeiss Axiovert 135M fluorescence microscope fitted with a Fluar 40X, NA: 1.3 oil immersion objective and coupled to a
- 25 Photometrics CH250 charged coupled device (CCD) camera. The cells were illuminated with a 100 W HBO arc lamp. In the light path was a  $470\pm 20$  nm excitation filter, a 510 nm dichroic mirror and a  $515\pm 15$  nm emission filter for minimal image background. The cells were maintained at  $37^\circ\text{C}$  with a custom built stage heater.

Images were processed and analysed in the following manner:

- 30 Method 1: Stepwise procedure for quantitation of translocation of PKA:
- The image was corrected for dark current by performing a pixel-by-pixel subtraction of a dark image (an image taken under the same conditions as the actual image, except the camera shutter is not allowed to open).

The image was corrected for non-uniformity of the illumination by performing a pixel-by-pixel ratio with a flat field correction image (an image taken under the same conditions as the actual image of a uniformly fluorescent specimen).

The image histogram, i.e., the frequency of occurrence of each intensity value in the  
5 image, was calculated.

A smoothed, second derivative of the histogram was calculated and the second zero is determined. This zero corresponds to the inflection point of the histogram on the high side of the main peak representing the bulk of the image pixel values.

The value determined in step 4 was subtracted from the image. All negative values were  
10 discarded.

The variance (square of the standard deviation) of the remaining pixel values was determined. This value represents the "response" for that image.

Scintillation proximity assay (SPA) for independent quantitation of cAMP.

15 Method 2: Alternative method for quantitation of PKA redistribution:

The fluorescent aggregates are segmented from each image using an automatically found threshold based on the maximisation of the information measure between the object and background. The *a priori* entropy of the image histogram is used as the information measure.

20 The area of each image occupied by the aggregates is calculated by counting pixels in the segmented areas.

The value obtained in step 2 for each image in a series, or treatment pair, is normalised to the value found for the first (unstimulated) image collected. A value of zero (0) indicates no redistribution of fluorescence from the starting condition. A value of one (1)  
25 by this method equals full redistribution.

Cells were cultured in HAM F-12 medium as described above, but in 96-well plates. The medium was exchanged with  $\text{Ca}^{2+}$ -HEPES buffer including 100  $\mu\text{M}$  IBMX and the cells were stimulated with different concentrations of forskolin for 10 min. Reactions were stopped with addition of NaOH to 0.14 M and the amount of cAMP produced was  
30 measured with the cAMP-SPA kit, RPA538 (Amersham) as described by the manufacturer.

Manipulating intracellular levels of cAMP to test the PKAc-F64L-S65T-GFP fusion.

The following compounds were used to vary cAMP levels: Forskolin, an activator of adenylate cyclase; dbcAMP, a membrane permeable cAMP analog which is not degraded by phosphodiesterase; IBMX, an inhibitor of phosphodiesterase.

CHO cells stably expressing the PKAc-F64L-S65T-GFP, showed a dramatic

- 5 translocation of the fusion protein from a punctate distribution to an even distribution throughout the cytoplasm following stimulation with 1  $\mu$ M forskolin (n=3), 10  $\mu$ M forskolin (n=4) and 50  $\mu$ M forskolin (n=4) (Fig 1), or dbcAMP at 1mM (n=6).

Fig. 2 shows the progression of response in time following treatment with 1  $\mu$ M forskolin.

- Fig. 3 gives a comparison of the average temporal profiles of fusion protein redistribution  
10 and a measure of the extent of each response to the three forskolin concentrations (Fig. 3A, E, B), and to 1 mM dbcAMP (fig 3C) which caused a similar but slower response, and to addition of 100  $\mu$ M IBMX (n=4, Fig. 3D) which also caused a slow response, even in the absence of adenylate cyclase stimulation. Addition of buffer (n=2) had no effect (data not shown).

- 15 As a control for the behaviour of the fusion protein, F64L-S65T-GFP alone was expressed in CHO cells and these were also given 50  $\mu$ M forskolin (n=5); the uniform diffuse distribution characteristic of GFP in these cells was unaffected by such treatment (data not shown).

- The forskolin-induced translocation of PKAc-F64L-S65T-GFP showed a dose-response  
20 relationship (Fig 4 and 6), see quantitative procedures above.

#### Reversibility of PKAc-F64L-S65T-GFP translocation.

The release of the PKAc probe from its cytoplasmic anchoring hotspots was reversible.

- Washing the cells repeatedly (5-8 times) with buffer after 10 $\mu$ M forskolin treatment  
25 completely restored the punctate pattern within 2-5 min (n=2, Fig. 3E). In fact the fusion protein returned to a pattern of fluorescent cytoplasmic aggregates virtually indistinguishable from that observed before forskolin stimulation.

- To test whether the return of fusion protein to the cytoplasmic aggregates reflected a decreased [cAMP], cells were treated with a combination of 10  $\mu$ M forskolin and 100  $\mu$ M  
30 IBMX (n=2) then washed repeatedly (5-8 times) with buffer containing 100  $\mu$ M IBMX (Fig. 3F). In these experiments, the fusion protein did not return to its prestimulatory localisation after removal of forskolin.

Testing the PKA-F64L-S65T-GFP probe with physiologically relevant agents.

- To test the probe's response to receptor activation of adenylate cyclase, BHK cells stably transfected with the glucagon receptor and the PKA-F64L-S65T-GFP probe were exposed to glucagon stimulation. The glucagon receptor is coupled to a  $G_s$  protein which activates adenylate cyclase, thereby increasing the cAMP level. In these cells, addition of 100 nM glucagon ( $n=2$ ) caused the release of the PKA-F64L-S65T-GFP probe from the cytoplasmic aggregates and a resulting translocation of the fusion protein to a more even cytoplasmic distribution within 2-3 min (Fig. 3G). Similar but less pronounced effects were seen at lower glucagon concentrations ( $n=2$ , data not shown). Addition of buffer ( $n=2$ ) had no effect over time (data not shown).
- 10 Transiently transfected CHO cells expressing hAR $\alpha$ 2a and the PKA-F64L-S65T-GFP probe were treated with 10  $\mu$ M forskolin for 7.5 minutes, then, in the continued presence of forskolin, exposed to 10  $\mu$ M norepinephrine to stimulate the exogenous adrenoreceptors, which couple to a  $G_i$  protein, which inhibit adenylate cyclase. This treatment led to reappearance of fluorescence in the cytoplasmic aggregates indicative of a decrease in  $[cAMP]_i$  (Fig. 3H).

Fusion protein translocation correlated with  $[cAMP]_i$

- As described above, the time it took for a response to come to completion was dependent on the forskolin dose (Fig. 5) In addition the degree of responses was also dose-dependent. To test the PKA-F64L-S65T-GFP fusion protein translocation in a semi high through-put system, CHO cells stably transfected with the PKA-F64L-S65T-GFP fusion was stimulated with buffer and 5 increasing doses of forskolin ( $n=8$ ). Using the image analysis algorithm described above (Method 1), a dose-response relationship was observed in the range from 0.01-50  $\mu$ M forskolin (Fig. 6). A half-maximal stimulation was observed at about 2  $\mu$ M forskolin. In parallel, cells were stimulated with buffer and 8 increasing concentrations of forskolin ( $n=4$ ) in the range 0.01-50  $\mu$ M. The amount of cAMP produced was measured in an SPA assay. A steep increase was observed between 1 and 5  $\mu$ M forskolin coincident with the steepest part of the curve for fusion protein translocation (also Fig. 6).

30

### **EXAMPLE 2 Probe for detection of PKC activity**

Construction of PKC-GFP fusion:

The probe was constructed by ligating two restriction enzyme treated polymerase chain reaction (PCR) amplification products of the cDNA for murine PKC $\alpha$  (GenBank

Accession number: M25811) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) respectively. Taq® polymerase and the following oligonucleotide primers were used for PCR;

5'mPKC $\alpha$ :

5 TTggACACAAgCTTTggACACCCTCAggATATggCTgACgTTTACCCggCCAACg

3'mPKC $\alpha$ :

gTCATCTTCTCgAgTCTTTCAggCgCgCCCTACTgCACTTTgCAAATTgggTgC,

5'F64L-S65T-GFP:

TTggACACAAgCTTTggACACggCgCgCCATgAgTAAAggAgAAgAACTTTTC,

10 3'F64L-S65T-GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT.

The hybrid DNA strand was inserted into the pZeoSV® mammalian expression vector as a HindIII-XhoI cassette as described in example 1.

- 15 BHK cells expressing the human M1 receptor under the control of the inducible metallothionein promoter and maintained with the dihydrofolate reductase marker were transfected with the PKC $\alpha$ -F64L-S65T-GFP probe using the calcium phosphate precipitate method in HEPES buffered saline (HBS [pH 7.10]). Stable transfectants were selected using 1000  $\mu$ g Zeocin®/ml in the growth medium (DMEM with 1000 mg
- 20 glucose/l, 10 % foetal bovine serum (FBS), 100  $\mu$ g penicillin-streptomycin mixture ml<sup>-1</sup>, 2 mM l-glutamine). The hM1 receptor and PKC $\alpha$ -F64L-S65T-GFP fusion protein were maintained with 500 nM methotrexate and 500  $\mu$ g Zeocin®/ml respectively. 24 hours prior to any experiment, the cells were transferred to HAM F-12 medium with glutamax, 100  $\mu$ g penicillin-streptomycin mixture ml<sup>-1</sup> and 0.3 % FBS. This medium relieves
- 25 selection pressure, gives a low induction of signal transduction pathways and has a low autofluorescence at the relevant wavelength enabling fluorescence microscopy of cells straight from the incubator.

Method 1: Monitoring the PKC $\alpha$  activity in real time:

- 30 Digital images of live cells were gathered using a Zeiss Axiovert 135M fluorescence microscope fitted with a 40X, NA: 1.3 oil immersion objective and coupled to a Photometrics CH250 charged coupled device (CCD) camera. The cells were illuminated with a 100 W arc lamp. In the light path was a 470 $\pm$ 20 nm excitation filter, a 510 nm dichroic mirror and a 515 $\pm$ 15 nm emission filter for minimal image background. The cells
- 35 were kept and monitored to be at 37°C with a custom built stage heater.

Images were analyzed using the IPLab software package for Macintosh.

Upon stimulation of the M1-BHK cells, stably expressing the PKC $\alpha$ -F64L-S65T-GFP fusion, with carbachol we observed a dose-dependent transient translocation from the cytoplasm to the plasma membrane (Fig. 7a,b,c). Simultaneous measurement of the  
5 cytosolic free calcium concentration shows that the carbachol-induced calcium mobilisation precedes the translocation (Fig. 8).

Stepwise procedure for quantification of translocation of PKC $\alpha$ :

The image was corrected for dark current by performing a pixel-by-pixel subtraction of a dark image (an image taken under the same conditions as the actual image, except the  
10 camera shutter is not allowed to open).

The image was corrected for non-uniformity of the illumination by performing a pixel-by-pixel ratio with a flat field correction image (an image taken under the same conditions as the actual image of a uniformly fluorescent specimen).

A copy of the image was made in which the edges are identified. The edges in the image  
15 are found by a standard edge-detection procedure – convolving the image with a kernel which removes any large-scale unchanging components (i.e., background) and accentuates any small-scale changes (i.e., sharp edges). This image was then converted to a binary image by thresholding. Objects in the binary image which are too small to represent the edges of cells were discarded. A dilation of the binary image was  
20 performed to close any gaps in the image edges. Any edge objects in the image which were in contact with the borders of the image are discarded. This binary image represents the edge mask.

Another copy of image was made via the procedure in step 3. This copy was further processed to detect objects which enclose “holes” and setting all pixels inside the holes  
25 to the binary value of the edge, i.e., one. This image represents the whole cell mask.

The original image was masked with the edge mask from step 3 and the sum total of all pixel values is determined.

The original image was masked with the whole cell mask from step 4 and the sum total of all pixel values was determined.

30 The value from step 5 was divided by the value from step 6 to give the final result, the fraction of fluorescence intensity in the cells which was localized in the edges.



**EXAMPLE 3 Probes for detection of mitogen activated protein kinase Erk1 redistribution.**

Useful for monitoring signalling pathways involving MAPK, e.g. to identify compounds which modulate the activity of the pathway in living cells.

- 5 Erk1, a serine/threonine protein kinase, is a component of a signalling pathway that is activated by e.g. many growth factors.

Probes for detection of ERK-1 activity in real time within living cells:

The extracellular signal regulated kinase (ERK-1, a mitogen activated protein kinase, MAPK) is fused N- or C-terminally to a derivative of GFP. The resulting fusions

- 10 expressed in different mammalian cells are used for monitoring *in vivo* the nuclear translocation, and thereby the activation, of ERK1 in response to stimuli that activate the MAPK pathway.

The human Erk1 gene (GenBank Accession number: X60188) was amplified using PCR according to standard protocols with primers

- 15 Erk1-top

5'-TAGAATTCAACCATGGCGGCGGCGGCGGCG-3'

and Erk1-bottom/+stop

5'-TAGGATCCCTAGGGGGCCTCCAGCACTCC-3'.

- The PCR product was digested with restriction enzymes EcoR1 and BamH1, and ligated  
20 into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with EcoR1 and BamH1. This produces an EGFP-Erk1 fusion (SEQ ID NOs: 5 and 6) under the control of a CMV promoter.

- The plamid containing the EGFP-Erk1 fusion was transfected into HEK293 cells employing the FUGENE transfection reagent (Boehringer Mannheim). Prior to  
25 experiments the cells were grown to 80%-90% confluency 8 well chambers in DMEM with 10% FCS. The cells were washed in plain HAM F-12 medium (without FCS), and then incubated for 30-60 minutes in plain HAM F-12 (without FCS) with 100 micromolar PD98059, an inhibitor of MEK1, a kinase which activates Erk1; this step effectively empties the nucleus of EGFP-Erk1. Just before starting the experiment, the HAM F-12  
30 was replaced with Hepes buffer following a wash with Hepes buffer. This removes the PD98059 inhibitor; if blocking of MEK1 is still wanted (e.g. in control experiments), the inhibitor is included in the Hepes buffer.

The experimental setup of the microscope was as described in example 1.

- 60 images were collected with 10 seconds between each, and with the test compound  
35 added after image number 10.

Addition of EGF (1-100 nM) caused within minutes a redistribution of EGFP-Erk1 from the cytoplasm into the nucleus (Fig. 9a,b).

The response was quantitated as described below and a dose-dependent relationship between EGF concentration and nuclear translocation of EGFP-Erk1 was found (Fig.

- 5 9c,d). Redistribution of GFP fluorescence is expressed in this example as the change in the ratio value between areas in nuclear versus cytoplasmic compartments of the cell. Each time profile is the average of nuclear to cytoplasmic ratios from six cells in each treatment.

#### 10 **EXAMPLE 4 Probes for detection of Smad2 redistribution.**

Useful for monitoring signalling pathways activated by some members of the transforming growth factor-beta family, e.g. to identify compounds which modulate the activity of the pathway in living cells.

- Smad 2, a signal transducer, is a component of a signalling pathway that is induced by  
15 some members of the TGFbeta family of cytokines.

a) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers

Smad2-top

5'-GTGAATTGACCATGTCGTCCATCTTGCCATTC-3'

- 20 and Smad2-bottom/+stop

5'-GTGGTACCTTATGACATGCTTGAGCAACGCAC-3'.

The PCR product was digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-C1 (Clontech; Palo Alto; GenBank Accession number U55763) digested with EcoR1 and Acc65I. This produces an EGFP-Smad2 fusion (SEQ ID NOs: 7 and 8) under

- 25 the control of a CMV promoter.

b) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers

Smad2-top

5'-GTGAATTGACCATGTCGTCCATCTTGCCATTC-3'

- 30 and Smad2-bottom/-stop

5'-GTGGTACCCATGACATGCTTGAGCAACGCAC-3'.

The PCR product was digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with EcoR1 and Acc65I. This produces a Smad2-EGFP fusion (SEQ ID NOs:9 and 10) under

- 35 the control of a CMV promoter.

The plasmid containing the EGFP-Smad2 fusion was transfected into HEK293 cells, where it showed a cytoplasmic distribution. Prior to experiments the cells were grown in 8 well Nunc chambers in DMEM with 10% FCS to 80% confluence and starved overnight in HAM F-12 medium without FCS.

- 5 For experiments, the HAM F-12 medium was replaced with Hepes buffer pH 7.2. The experimental setup of the microscope was as described in example 1. 90 images were collected with 10 seconds between each, and with the test compound added after image number 5.

After serum starvation of cells, each nucleus contains less GFP fluorescence than the  
10 surrounding cytoplasm (Fig. 10a). Addition of TGFbeta caused within minutes a redistribution of EGFP-Smad2 from the cytoplasm into the nucleus (Fig. 10b).

The redistribution of fluorescence within the treated cells was quantified simply as the fractional increase in nuclear fluorescence normalised to the starting value of GFP fluorescence in the nucleus of each unstimulated cell and displayed a dose dependent  
15 change in response to TGFβ (fig. 10c).

#### **EXAMPLE 5 Probes for detection of VASP redistribution.**

Useful for monitoring signalling pathways involving rearrangement of cytoskeletal elements, e.g. to identify compounds which modulate the activity of the pathway in living cells. VASP, a phosphoprotein, is a component of cytoskeletal structures, which  
20 redistributes in response to signals that affect focal adhesions.

The human VASP gene (GenBank Accession number: Z46389) was amplified using PCR according to standard protocols with primers

VASP-top

5'-GGGAAGCTTCCATGAGCGAGACGGTCATC-3'

25 and VASP-bottom/+stop

5'-CCCGGATCCTCAGGGAGAACCCCGCTTC-3'.

The PCR product was digested with restriction enzymes Hind3 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Hind3 and BamH1. This produces an EGFP-VASP fusion (SEQ ID NOs:11 and 12) under  
30 the control of a CMV promoter.

The resulting plasmid was transfected into CHO cells expressing the human insulin receptor using the calcium-phosphate transfection method. Prior to experiments, cells were grown in 8 well Nunc chambers and starved overnight in medium without FCS.

Experiments are performed in a microscope setup as described in example 1. 10% FCS  
35 was added to the cells and images were collected. The EGFP-VASP fusion was

redistributed from a somewhat even distribution near the periphery into more localised structures, identified as focal adhesion points (Fig. 11).

**EXAMPLE 7 Probes for detection of NFkappaB redistribution.**

- 5 Useful for monitoring signalling pathways leading to activation of NFkappaB, e.g. to identify compounds which modulate the activity of the pathway in living cells.  
NFkappaB, an activator of transcription, is a component of signalling pathways that are responsive to a variety of inducers including cytokines, lymphokines, and some immunosuppressive agents.
- 10 a) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers  
NFkappaB-top  
5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC-3'  
and NFkappaB-bottom/+stop
- 15 5'-GTGGATCCTTAGGAGCTGATCTGACTCAGCAG-3'.  
The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Xho1 and BamH1. This produces an EGFP-NFkappaB fusion (SEQ ID NOs:13 and 14) under the control of a CMV promoter.
- 20 b) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers  
NFkappaB-top  
5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC-3'  
and NFkappaB-bottom/-stop
- 25 5'-GTGGATCCAAGGAGCTGATCTGACTCAGCAG-3'.  
The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with Xho1 and BamH1. This produces an NFkappaB-EGFP fusion (SEQ ID NOs: 15 and 16) under the control of a CMV promoter.
- 30 The resulting plasmids are transfected into a suitable cell line, e.g. Jurkat, in which the EGFP-NFkappaB probe and/or the NFkappaB-EGFP probe should change its cellular distribution from cytoplasmic to nuclear in response to activation of the signalling pathway with e.g. IL-1.

CHO cells stably expressing the insulin receptor and a human NFkB – GFP protein hybrid were stimulated with different concentrations of IL-1 for 1 hour, then washed with a hypoosmotic buffer (TRIS-base 10mM, MgCl<sub>2</sub> 2mM, PMSF(Phenyl methyl sulfonyl fluoride) 0.5mM, pH 7.4) and placed on the microscope. A sequence of images were  
 5 acquired during the addition of 0.05% Triton X-100 and subsequent gentle mixing after a short incubation period. The treatment causes the cell membranes to rupture leaving the fraction of NFkB-GFP that has translocated to the nucleus behind whereas the cytoplasmic amount of the probe leaves the cells more quickly and immediately becomes infinitely diluted in the surrounding medium (out of focus - this part of the total  
 10 fluorescence from the probe is thereby lost). At a defined time point before and after this treatment a total intensity value for the whole image was extracted. To normalize each experiment, the after value was divided by the before value, meaning that a higher ratio was found in cells where more NFkB had translocated to the nucleus and thereby contributed to the total fluorescence after permeabilisation. the actual data from such an  
 15 experiment run in duplicate is shown in Figure 18.

Conclusion: the present protocol is a good example of the possibility of revealing translocation of a fluorescent probe from the cytosol to the nucleus or translocation from the nucleus to the cytosol by using a measurement immediately before and after plasma membrane permeabilisation recorded as an image sequence.

20

### ***EXAMPLE 8 real-time redistribution of protein kinase C $\alpha$***

Measurement of the real-time redistribution of protein kinase C  $\alpha$  isoform-GFP fusion (PKC $\alpha$ -GFP, SEQ ID NOs: 3 and 4) in response to carbamylcholine stimulation of the muscarinic M1 receptor; 96 parallel redistribution measurements in microtiter plates.

25 BHK cells were stably expressing a recombinant human muscarinic type 1 receptor, under the selection with 500  $\mu$ g/ml Methotrexate, and also a PKC $\alpha$ -GFP construct (K $\alpha$ A 048), under the selection of 500 nM Zeocin. The cells were grown in 96-well plates (Packard ViewPlate, black with transparent bottom), washed and preincubated in a Hank's Buffered Salt solution (HBSS) without phenol red, with 20 mM HEPES and 5.5  
 30 mM glucose.

The plate was measured in a FLIPR™ (Fluorescence Imaging Plate Reader) from Molecular Devices. The 488 nm emission line from an argon ion laser, run at between 0.4 and 0.8 W output, was used to excite fluorescence from the GFP. Emission wavelengths were collected through a 510 to 565 nm band pass filter.

The cells were challenged with three doses of carbamylcholine, an M1 receptor agonist known from previous studies to give a microscopically detectable redistribution of the PKC $\alpha$ -GFP construct [(Almholt *et al.* 1997)]. Measurements were made every 10 seconds for 5 minutes. After data handling including normalisation of baseline fluorescence for the different wells, background subtraction and averaging the 6 wells used for each concentration the data presented in figure 14 were obtained. It can clearly be seen (Fig 12) that carbamylcholine gave a time- and dose-dependent, and transient, decrease in fluorescence very similar to the time- and dose-dependent profile seen in microscopic fluorescence measurements [(see Almholt *et al.* 1997)]. This experiment was repeated twice on the same batch of cells with similar results.

**EXAMPLE 9 real-time redistribution of cyclic-AMP dependent protein kinase catalytic subunit-GFP fusion**

Measurement of the real-time redistribution of cyclic-AMP dependent protein kinase catalytic subunit-GFP fusion (C-GFP<sup>LT</sup> SEQ ID NOs: 1 and 2) in response to forskolin stimulation of the adenylate cyclase; 96 parallel redistribution measurements in microtiter plates.

CHO cells were stably transfected with hybrid DNA for the PKA catalytic subunit-F64L+S65T GFP (C-GFP<sup>LT</sup>) fusion protein, and were typically under continuous selection with 1000  $\mu$ g/ml zeocin (Invitrogen). The cells were grown without selection for 2 days in 96-well plates (Packard ViewPlate, black with transparent bottom), washed and preincubated in a Hank's Buffered Salt solution (HBSS) without phenol red, with 20 mM HEPES and 5.5 mM glucose.

The plate was measured in a FLIPR<sup>TM</sup> (Fluorescence Imaging Plate Reader) from Molecular Devices. The 488 nm emission line from an argon ion laser, run at between 0.4 and 0.8 W output, was used to excite fluorescence from the GFP. Emission wavelengths were collected through a 510 to 565 nm band pass filter.

The cells were challenged with three doses of forskolin (Fig 13), an adenylate cyclase agonist known from previous studies to give a microscopically detectable redistribution of the C-GFP<sup>LT</sup> construct. Measurements were made every 10 seconds for over 6 minutes from the point of addition of forskolin. After data handling including normalisation of baseline fluorescence for the different wells, background subtraction and averaging the 6 wells used for each concentration the data presented below were obtained. It can clearly be seen in figure 15 that forskolin gave a time- and dose-dependent decrease in fluorescence very similar to the time- and dose-dependent profile seen in microscopic

fluorescence measurements. This experiment was repeated twice on the same batch of cells with similar results. As can be seen in figure 14, a more extensive dose-response test gives at hand that this method is both sensitive and reproducible enough to use as the basis for a high throughput screening assay.

#### 5 **EXAMPLE 10 cyclic-AMP dependent protein kinase catalytic subunit-GFP fusion**

Measurement of the redistribution response of cyclic-AMP dependent protein kinase catalytic subunit-GFP fusion (C-GFP<sup>LT</sup> SEQ ID NOs: 1 and 2) after forskolin stimulation of the adenylate cyclase; measurement of the change in total fluorescence upon

10 permeabilisation of agonist-treated cells.

CHO cells were stably transfected with hybrid DNA for the PKA catalytic subunit-F64L+S65T GFP (C-GFP<sup>LT</sup>) fusion protein, and were typically under continuous selection with 1000 µg/ml zeocin (Invitrogen). For the experiments reported here, cells were grown without selection to 90% confluence in 8-well tissue culture-treated Lab-Tek®

15 chambered coverglass units (chambers, obtained from Nunc, Inc. Illinois, USA).

Immediately prior to the experiment growth medium was washed from the cells and replaced with 200 µl HEPES buffer per well.

For the results reported here, chambers were measured using a cooled CCD camera (KAF1400 chip, Photometrics Ltd., USA) attached to an inverted microscope (Diaphot  
20 300, Nikon, Japan) equipped with a x40 oil-immersion Fluor lens, NA 1.4. Cells were illuminated with 450-490 nm light from a 50 W HBO lamp, and emitted light collected between 510-560 nm.

The cells were challenged with four doses of forskolin, an adenylate cyclase agonist known from previous studies to give a microscopically detectable redistribution of the C-GFP<sup>LT</sup> construct. Images were collected at 10-second intervals for a period of 10 minutes  
25 for each treatment. Six minutes after the addition of forskolin or buffer control, Triton-X100 was added to a final concentration of 0.1%. The detergent releases freely mobile C-GFP<sup>LT</sup> from the cells. The change in fluorescence resulting from this loss was measured after 1 minute of equilibration. After data handling including background  
30 subtraction and normalisation to pre-detergent values, the data presented in figure 16 were obtained. Permeabilisation caused decreases in fluorescence, the magnitude of which were dependent on the forskolin treatments. This experiment was repeated twice on the same batch of cells with similar results.

**EXAMPLE 11 Probes for detection of PKC $\beta$ 1 redistribution.**

Useful for monitoring signalling pathways involving Protein Kinase C, e.g. to identify compounds which modulate the activity of the pathway in living cells.

PKC $\beta$ 1, a serine/threonine protein kinase, is closely related to PKC $\alpha$  and

- 5 PKC $\beta$ 2 but not identical; it is a component of a signalling pathway which is activated by elevation of intracellular calcium concomitant with an increase in diacylglycerol species.

a) The human PKC $\beta$ 1 gene (GenBank Accession number: X06318) was amplified

- 10 using PCR according to standard protocols with primers

PKC $\beta$ 1-top

GTCTCGAGGCAAGATGGCTGACCC

and PKC $\beta$ 1-bottom

GTGGATCCCTACACATTAATGACAACTCTGGG.

- 15 The PCR product was digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Xho1 and BamH1. This produces an EGFP-PKC $\beta$ 1 fusion (SEQ ID NOs: 17 and 18) under the control of a CMV promoter.

b) CHO cells stably expressing the human insulin receptor and human PKC beta 1

- 20 labeled with EGFP were investigated in the microscope. A dose-response was created where a set of cells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 4 min of stimulation.

It can be seen in figure 16 that using microscopic measurements, redistribution of human PKC beta 1 – EGFP can only be detected if a subregion of each cell is analysed. The

- 25 event is clearly visible when the image series is viewed as a movie but if the whole image changes in fluorescence or the changes in fluorescence in entire cells are analysed the redistribution cannot be detected.

CHO cells stably expressing the human insulin receptor and human PKC beta 1 labelled with EGFP were investigated in the FLIPR™. A dose-response was created where six

- 30 separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 5 min of stimulation. As shown in figure 17 redistribution of human PKC beta 1 – EGFP can be detected in the FLIPR™ instrument despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated and imaged with a resolution far below what is needed to resolve single cells or
- 35 subcellular compartments. This phenomenon can clearly not be predicted from the



microscope data in Figure 16. Based on these observations it is clear that a screening assay can be established in the FLIPR™ instrument. It might even be possible to establish a high throughput screening assay with further optimisation.

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## CLAIMS

1. A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising  
5 recording variation in spatially distributed light emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, as a change in light intensity measured by an instrument  
10 designed for the measurement of changes in fluorescence intensity.
2. A method according to claim 1, wherein the quantitative information which is indicative of the degree of the cellular response to the influence or the result of the influence on the subcellular component is extracted from the recorded variation according to a  
15 predetermined calibration based on responses or results, recorded in the same manner, to known degrees of a relevant specific influence.
3. A method according to claims 1 or 2, wherein the influence comprises contact between the mechanically intact or permeabilised living cells and a chemical substance and/or incubation of the mechanically intact or permeabilised living cells with a chemical  
20 substance.
4. A method according to any of claims 1-3, wherein the cells comprise a group of cells contained within a spatial limitation.
5. A method according to any of claims 1-4, wherein the cells comprise multiple groups of cells contained within multiple spatial limitations.
- 25 6. A method according to any of claims 1-5, wherein the cells comprise multiple groups of cells that are qualitatively the same but are subjected to different influences.
7. A method according to any of claims 1-5, wherein the cells comprise multiple groups of cells that are qualitatively different but are subjected to the same influence.
8. A method according to any of claims 1-7, wherein multiple spatial limitations are  
30 measured simultaneously by means of a one- or two-dimensional array detector, whereby the multiple spatial limitations are imaged onto the array detector such that

discrete subsets of the detecting units (pixels) in the array detector measure the signal from one and only one of the multiple spatial limitations, the signal from any one spatial limitation being the combined signal from those pixels that receive the image from one of the spatial limitations.

- 5 9. A method according to claim 8, wherein the detector is a linear diode array.
10. A method according to claim 8, wherein the detector is a video camera.
11. A method according to claim 8, wherein the detector is a charge transfer device.
12. A method according to claim 8, wherein the charge transfer device is a charge-coupled device.
- 10 13. A method according to any of claims 1-12, wherein all of the multiple spatial limitations are simultaneously illuminated during the measurement operation.
14. A method according to any of claims 1-12, wherein the individual spatial limitations are singly illuminated only during the time period in which they are being measured.
15. A method according to any of claims 1-14, wherein the illumination is provided by a
- 15 15. A method according to any of claims 1-14, wherein the illumination is provided by a laser which is scanned in a raster fashion over some or all of the spatial limitations being measured, the scanning taking place at a rate substantially faster than the measurement process such that the illumination appears to the measurement process to be continuous in time and spatially uniform over the region being measured.
16. A method according to any of claims 1-15, wherein the spatial limitations are spatial
- 20 16. A method according to any of claims 1-15, wherein the spatial limitations are spatial limitations arranged in one or more arrays on a common carrier.
17. A method according to claim 16, wherein the spatial limitations are wells in a plate of microtiter type.
18. A method according to any of claims 1-17, wherein the spatial limitations are domains defined on a substrate on which the cells are present.
- 25 19. A method according to claim 18, wherein the domains are domains established by the presence of the cells on the substrate in a pattern defining the domains.
20. A method according to claim 18, wherein the domains are domains established by the spatial pattern of the influence as it is applied to or contacted with the cells.

21. A method according to any of claims 1-20, wherein the recording is performed at a series of points in time, in which the application of the influence occurs at some time after the first time point in the series of recordings, the recording being performed, e.g., with a predetermined time spacing of from 0.1 seconds to 1 hour, preferably from 1 to 60  
5 seconds, more preferably from 1 to 30 seconds, in particular from 1 to 10 seconds, over a time span of from 1 second to 12 hours, such as from 10 seconds to 12 hours, e.g., from 10 seconds to one hour, such as from 60 seconds to 30 minutes or 20 minutes.
22. A method according to claim 21, wherein the recording is made at two points in time, one point being before, and the other point being after the application of the influence.
- 10 23. A method according to any of claims 1-22, wherein the cells are fixed at a point in time after the application of the influence at which the response has been predetermined to be significant, and the recording is made at an arbitrary later time.
24. A method according to any of claims 1-23, wherein the redistribution results in quenching of fluorescence, the quenching being measured as a decrease in the intensity  
15 of the fluorescence.
25. A method according to any of claims 1-24, wherein the redistribution results in energy transfer, the energy transfer being measured as a change in the intensity of the luminescence.
26. A method according to any of claims 1-24, wherein the illumination necessary to  
20 excite fluorescence is non-homogeneous such that the redistribution results in a greater or lesser number of fluorescent molecules being excited, the result being measured as a change in fluorescent intensity.
27. A method according to any of claims 1-24, wherein the intensity of the light being recorded is a function of the fluorescence lifetime, polarisation, wavelength shift, or other  
25 property which is modulated as a result of the underlying cellular response.
28. A method according to any of claims 1-27, wherein the light to be measured passes through a filter which selects the desired component of the light to be measured and rejects other components.

29. A method according to any of claims 1-28, wherein the fluorescence comes from a fluorophore encoded by and expressed from a nucleotide sequence harboured in the cells.

30. A method according to any of claims 1-28, wherein the fluorescence comes from a fluorophore introduced into the cells by any or various techniques for the bulk loading of material into cells such as transfection, incubation, scrape loading, electroporation.

31. A method according to any of the preceding claims, wherein the fluorescence comes from a luminescent polypeptide, such as GFP.

32. A method according to any of claims 1-31, wherein the cells are selected from the group consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; and vertebrate cells, such as mammalian cells.

33. A method according to claim 32, wherein the mechanically intact or permeabilised living cells are mammalian cells which, during the time period over which the influence is observed, are incubated at a temperature of 30°C or above, preferably at a temperature of from 32°C to 39°C, more preferably at a temperature of from 35°C to 38°C, and most preferably at a temperature of about 37°C.

34. A method according to any of the preceding claims, wherein the nucleic acid construct is a DNA construct with a sequence selected from the group consisting of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15 and 17 or is a variant thereof capable of encoding the same fusion polypeptide or a fusion polypeptide which is biologically equivalent thereto.

35. A method according to any of claims 1-34, used as a screening program.

36. A method according claim 35, wherein the method is a screening program for the identification of a biologically active substance that directly or indirectly affects an intracellular signalling pathway and is potentially useful as a medicament, wherein the result of the individual measurement of each substance being screened which indicates its potential biological activity is based on measurement of the redistribution of spatially resolved luminescence in living cells and which undergoes a change in distribution upon activation of an intracellular signalling pathway.

37. A method according to claim 35, wherein the method is a screening program for the identification of a biologically toxic substance as defined herein that exerts its toxic effect

by interfering with an intracellular signalling pathway, wherein the result of the individual measurement of each substance being screened which indicates its potential biologically toxic activity is based on measurement of the redistribution of said fluorescent probe in living cells and which undergoes a change in distribution upon activation of an  
5 intracellular signalling pathway.

38. A method according to any of claims 1-37 wherein a fluorescent probe is used in back-tracking of signal transduction pathways as defined herein.

39. A set of data relating to an influence on a cellular response in mechanically intact or permeabilised living cells, obtained by recording variation in spatially distributed light  
10 emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, as a change in light intensity measured by an instrument designed for the measurement of changes in  
15 fluorescence intensity.



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Figure 1

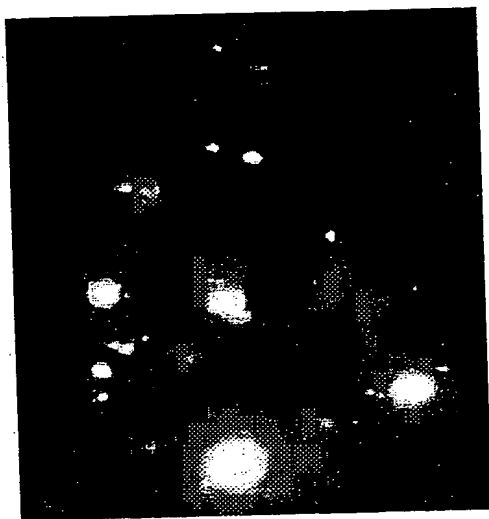


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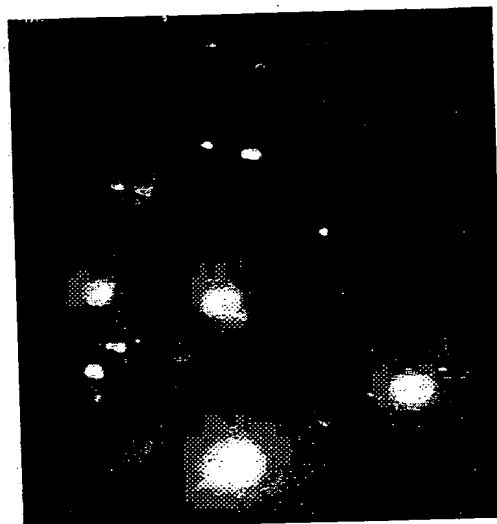


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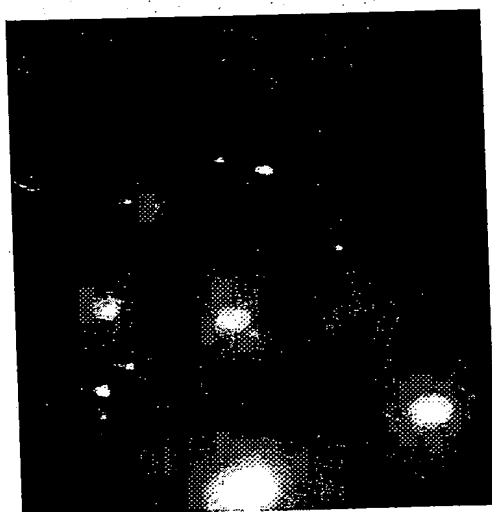


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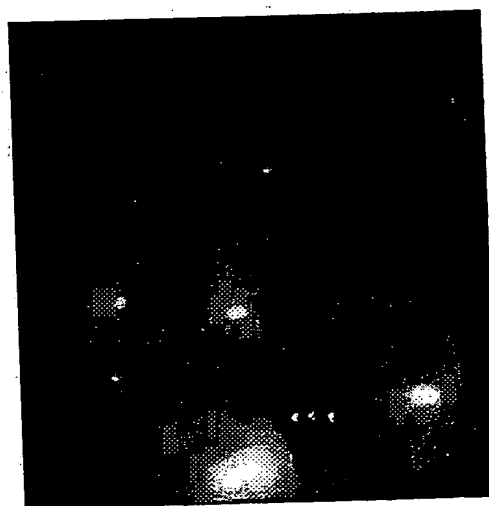


Fig. 1 d

Fig. 1

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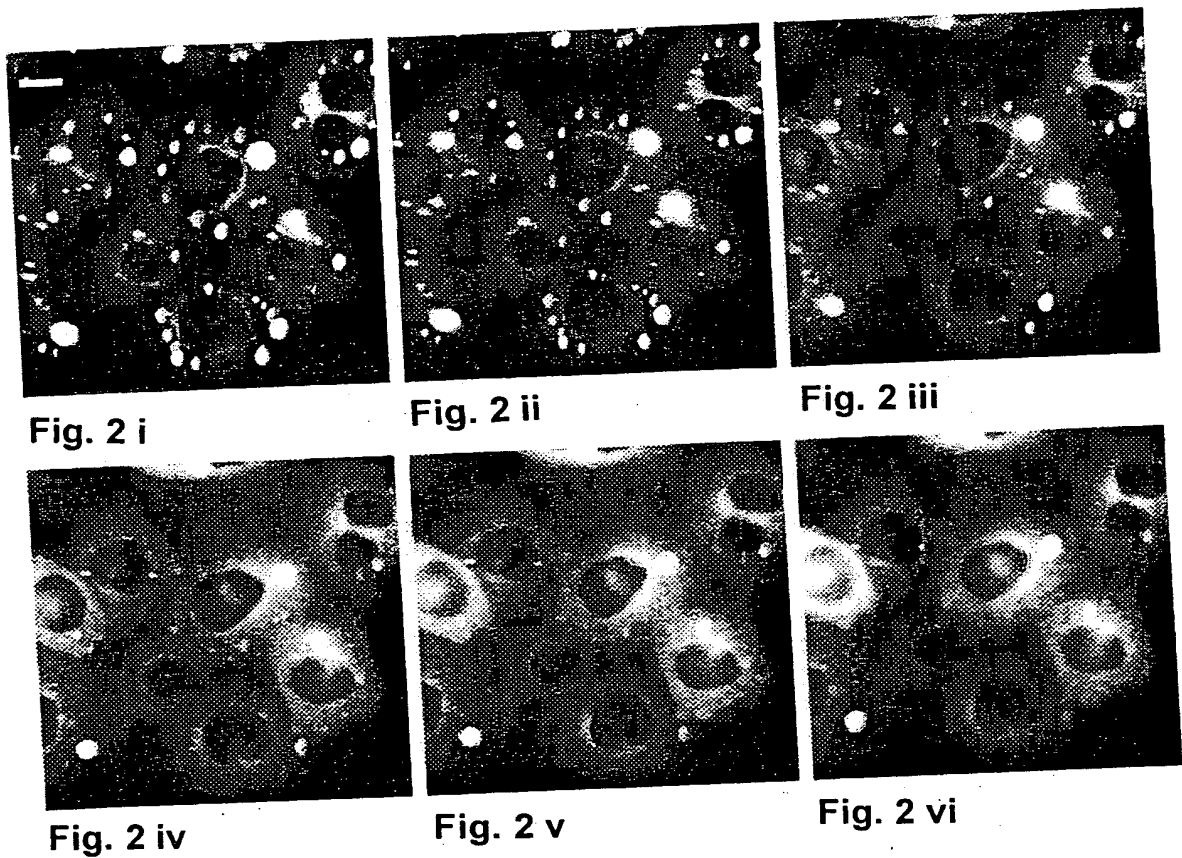


Fig. 2

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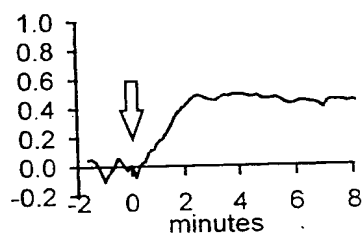


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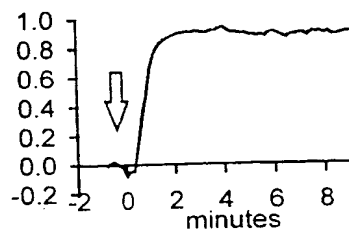


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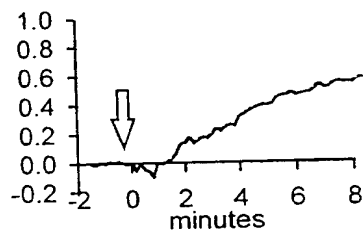


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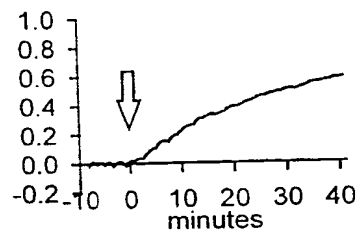


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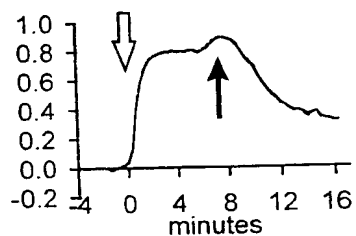


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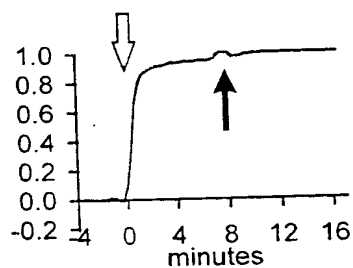


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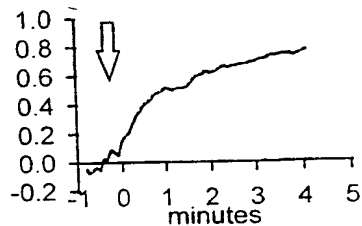


Fig. 3 G

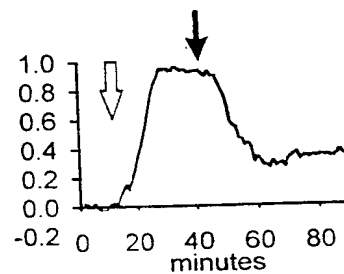


Fig. 3 H

Fig. 3

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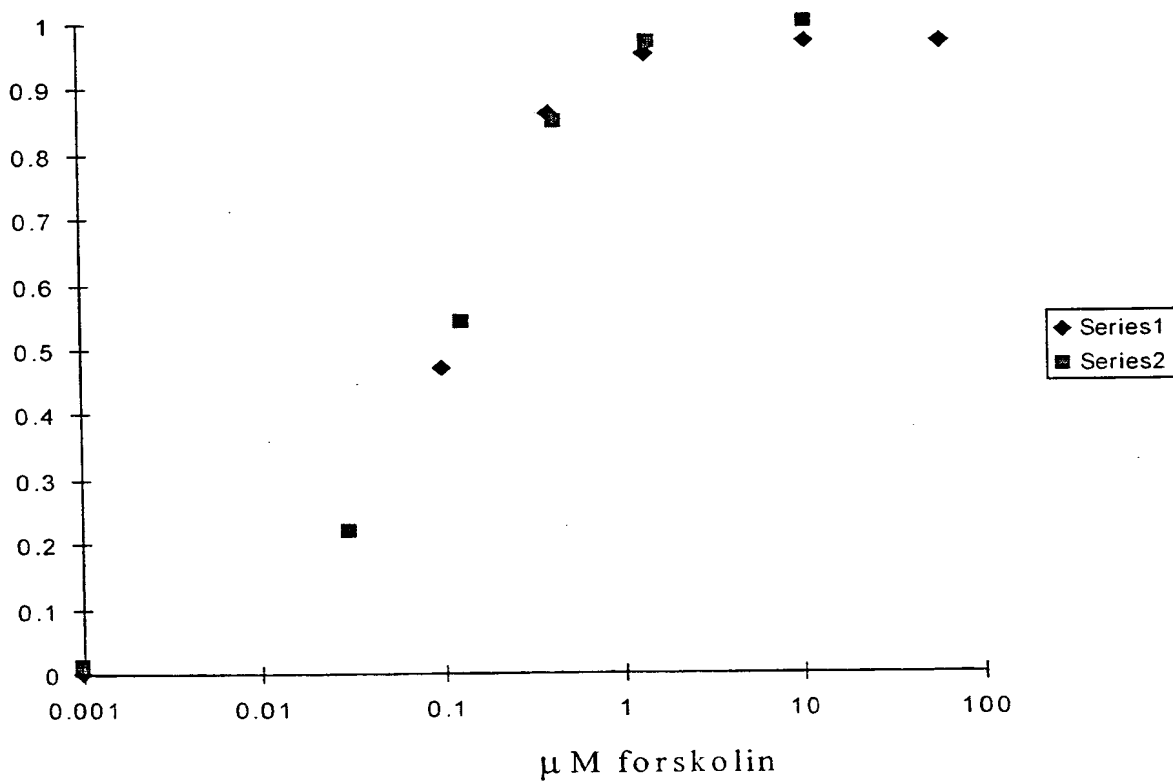


Fig. 4

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[forskolin] $\mu$ M	$t_{1/2\max}$ / s	$t_{\max}$ / s
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10	69 $\pm$ 14	224 $\pm$ 47
50	47 $\pm$ 10	125 $\pm$ 28

Fig. 5

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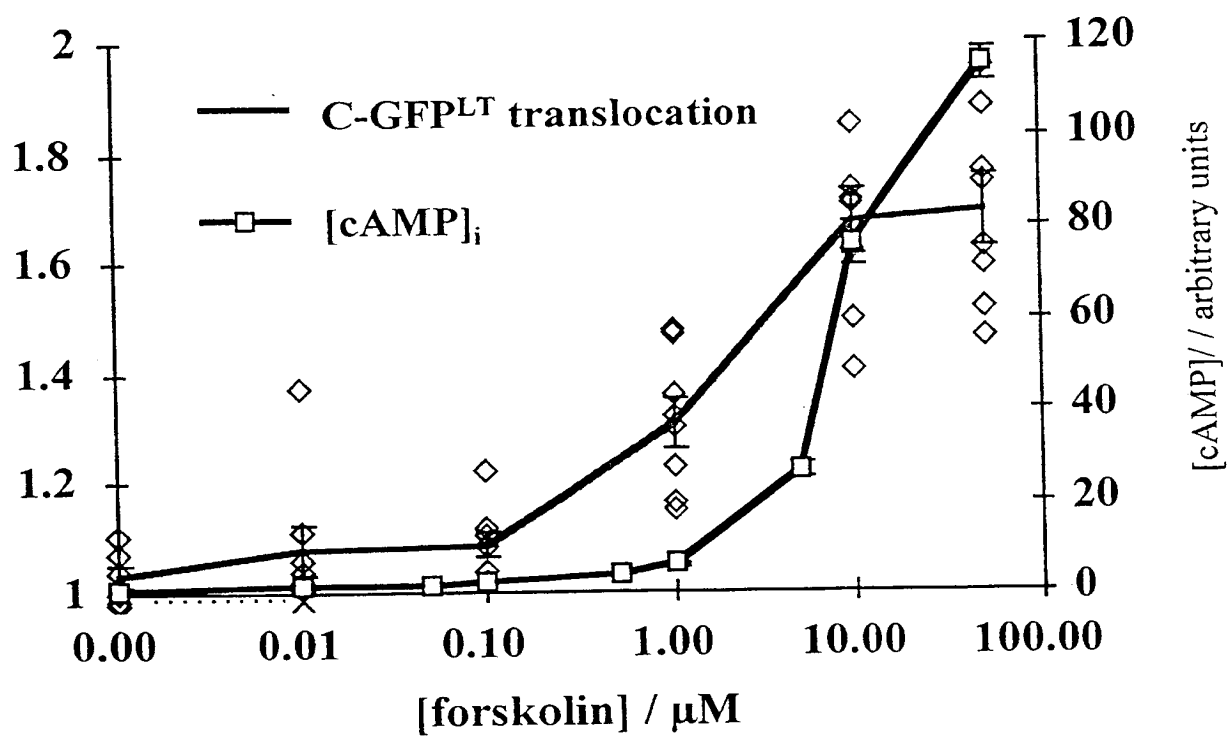


Fig. 6

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Fig. 7 a

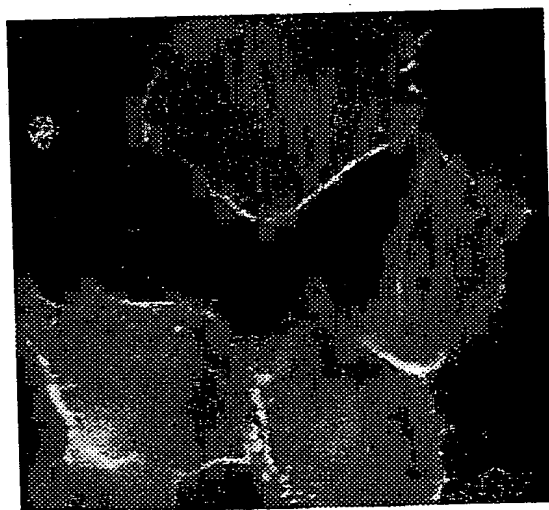


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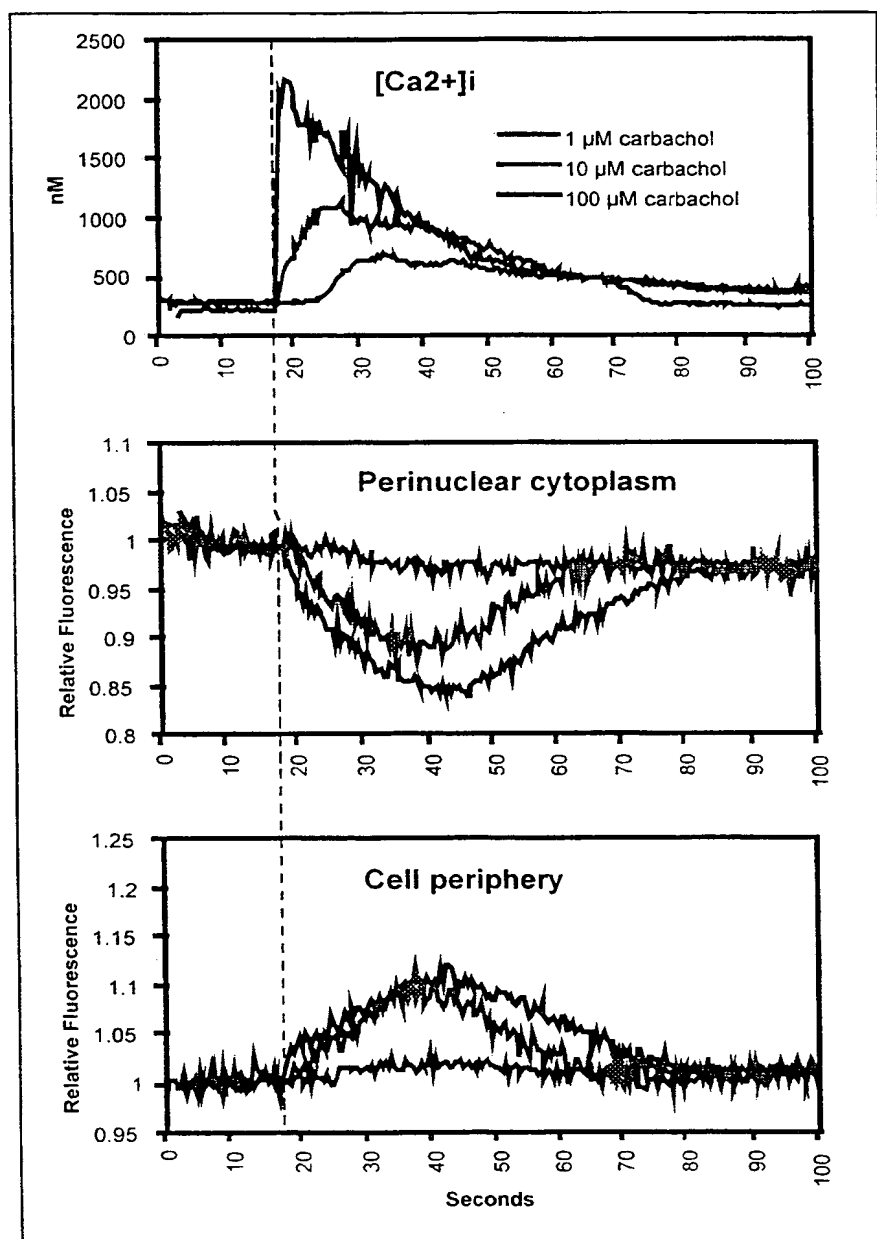


Fig. 7 c

**Fig. 7**

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**Fig. 8**

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Fig. 9 a

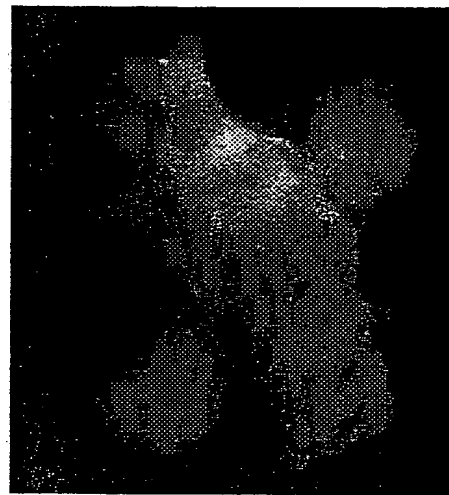


Fig. 9 b

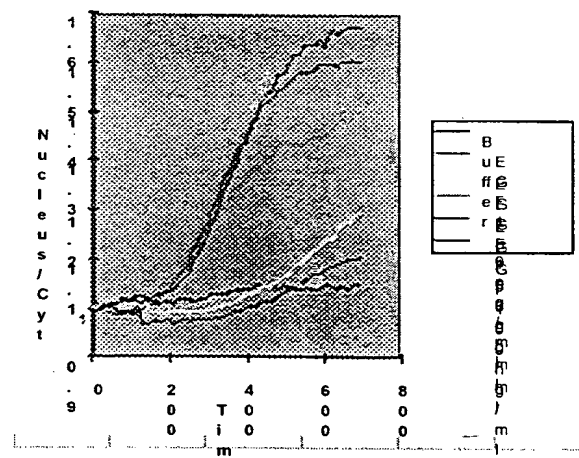


Fig. 9 c

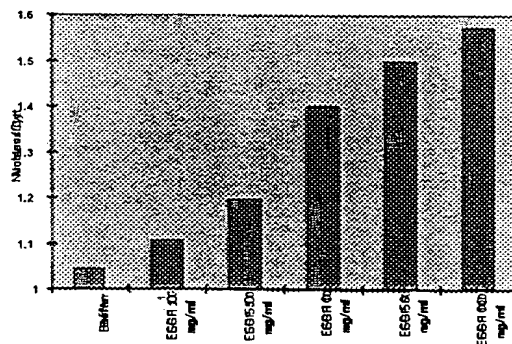


Fig. 9 d

Fig. 9

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Fig. 10 a



Fig. 10 b

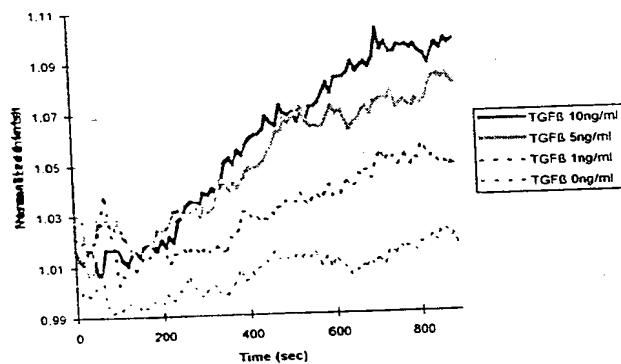
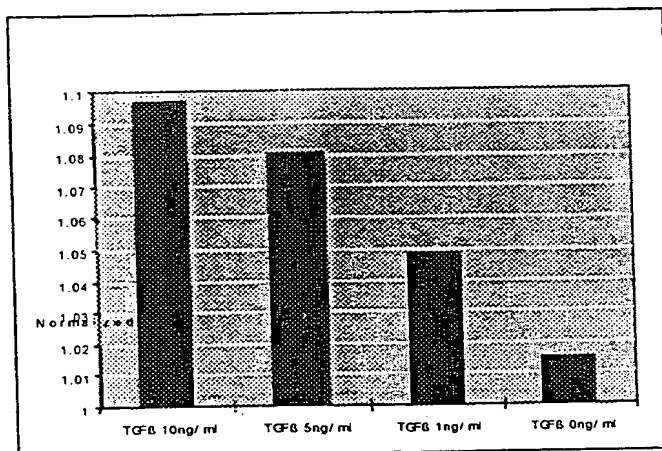


Fig. 10 c



**Fig. 10**  
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**Fig. 11**  
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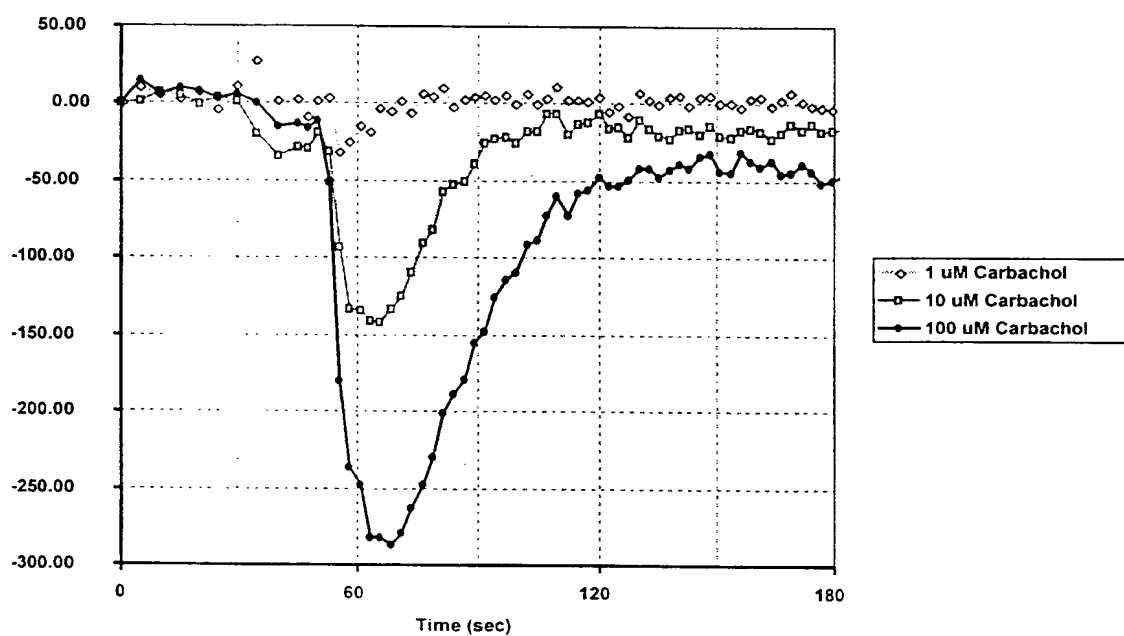


Fig. 12

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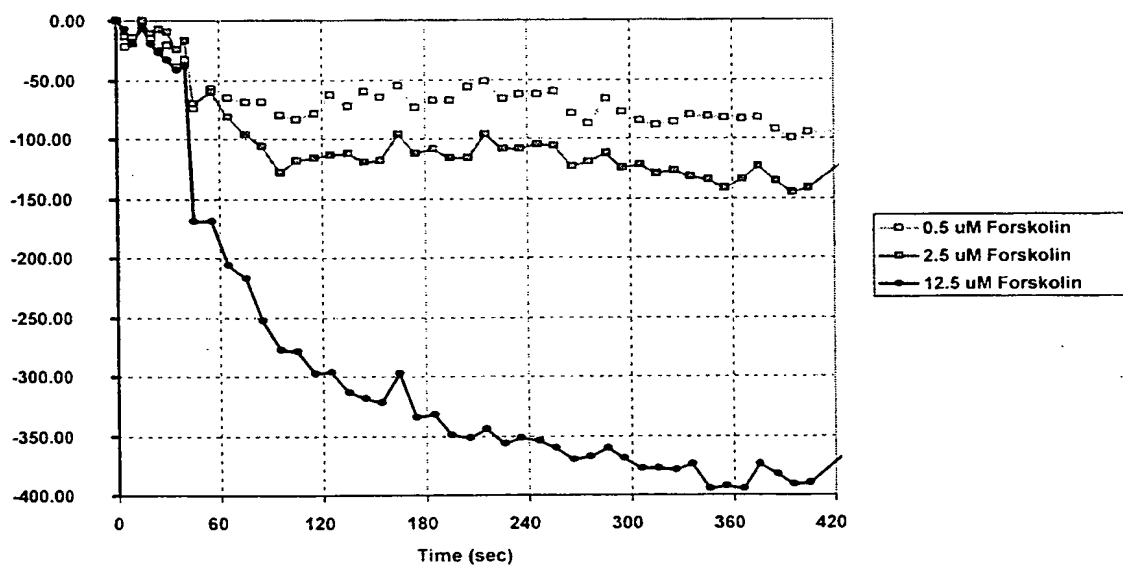
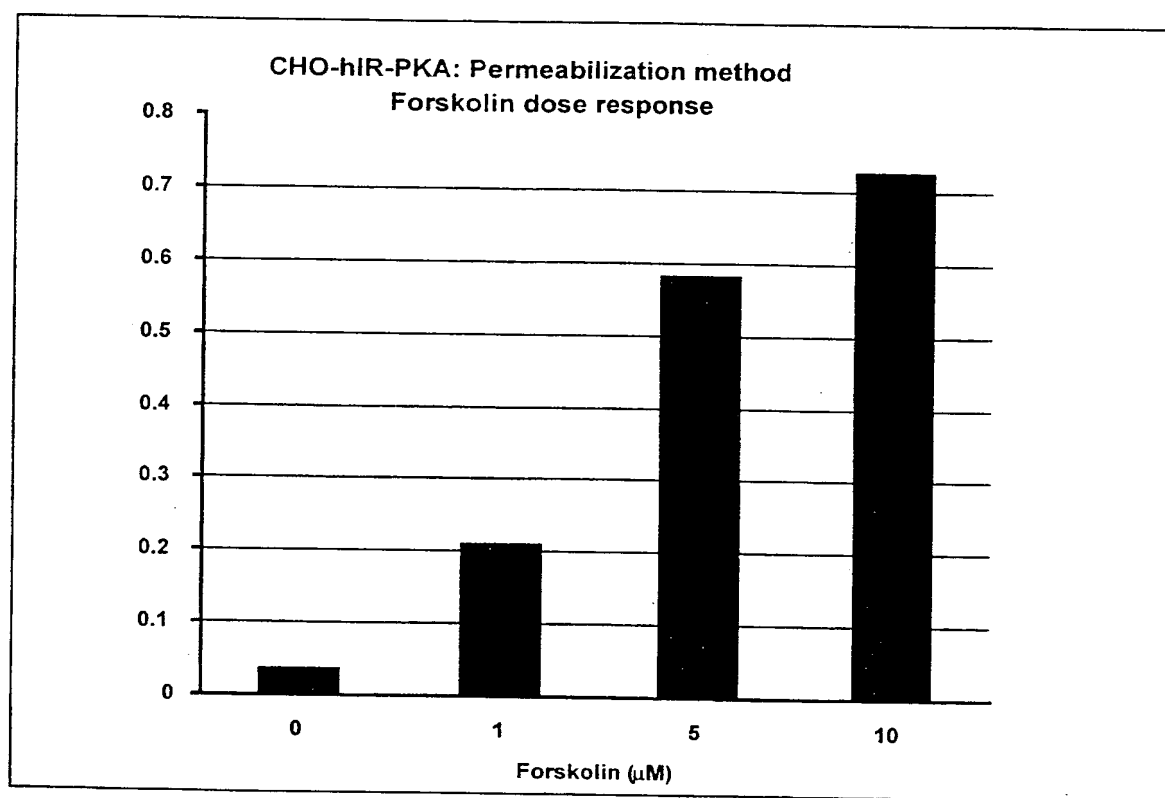


Fig. 13

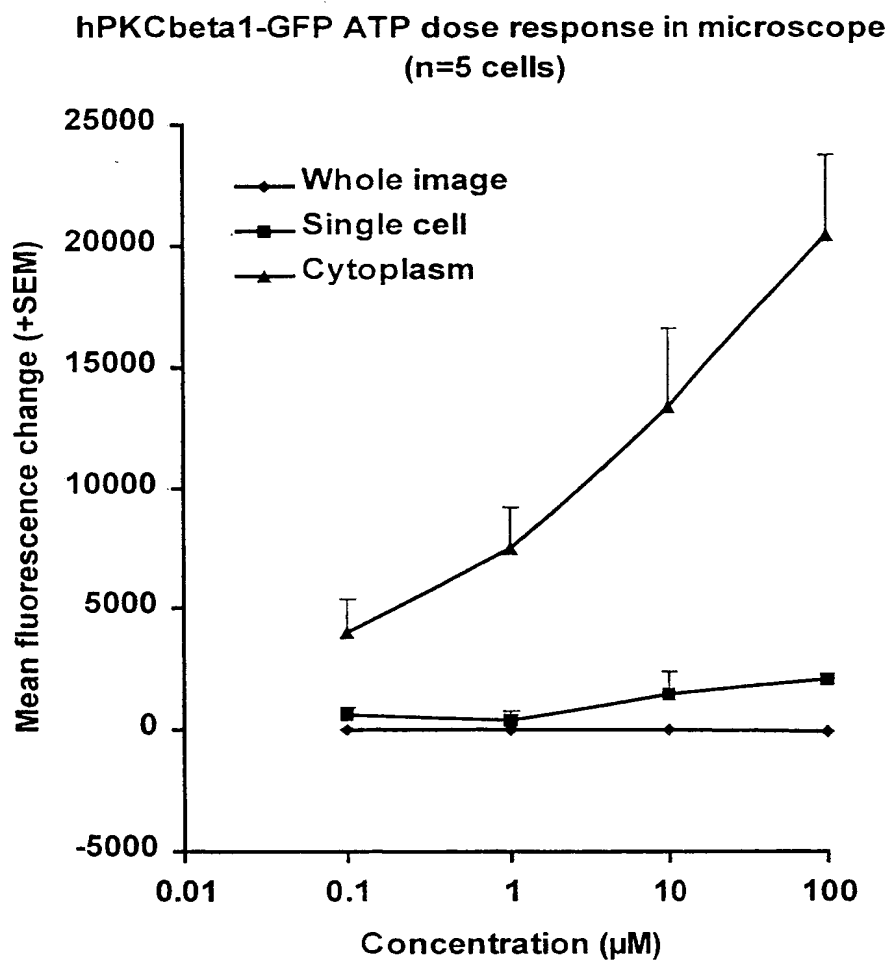
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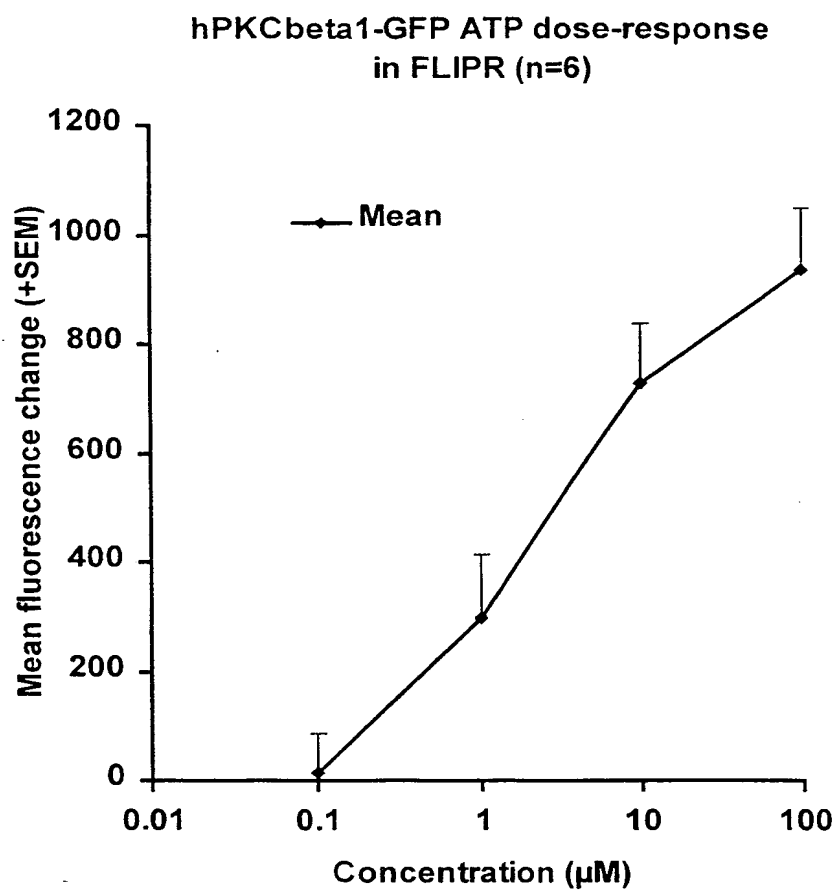
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**Fig. 15**  
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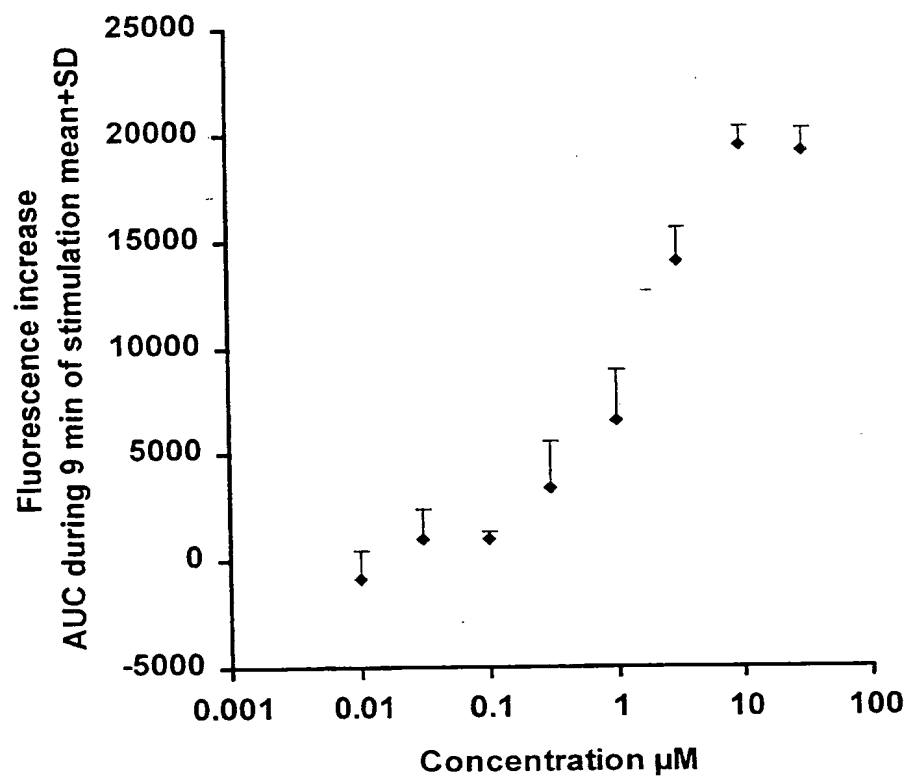
**Fig. 16**

SUBSTITUTE SHEET (RULE 26)



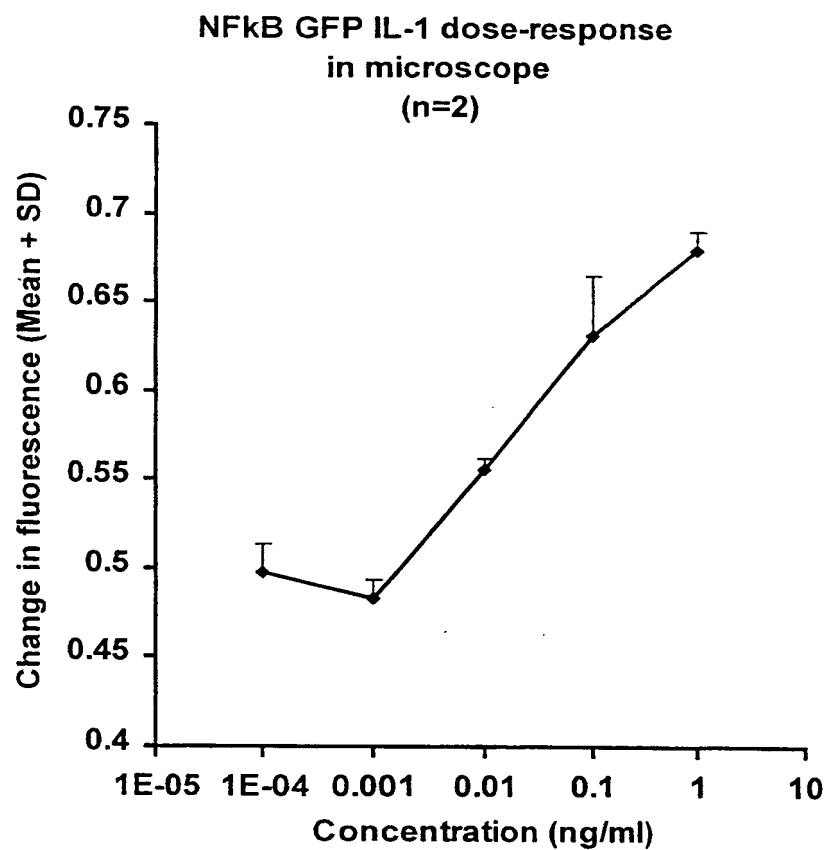
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cPKA BioST Forskolin dose-response  
on FLIPR (n=6).

**Fig. 17**

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**Fig. 18**

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## SEQUENCE LISTING

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<213> Aequorea victoria and mouse

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Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro Lys Asn Glu Ser	
195 200 205	
aaa cag aaa acc aaa acc atc cgc tcc aac ctg aat cct cag tgg aat	672
Lys Gln Lys Thr Lys Thr Ile Arg Ser Asn Leu Asn Pro Gln Trp Asn	
210 215 220	
gag tcc ttc acg ttc aaa tta aaa cct tca gac aaa gac cgg cga ctg	720
Glu Ser Phe Thr Phe Lys Leu Lys Pro Ser Asp Lys Asp Arg Arg Leu	
225 230 235 240	
tct gta gaa atc tgg gac tgg gat cgg acg act cgg aat gac ttc atg	768
Ser Val Glu Ile Trp Asp Trp Asp Arg Thr Thr Arg Asn Asp Phe Met	
245 250 255	
gga tcc ctt tcc ttt ggt gtc tca gag cta atg aag atg ccg gcc agt	816
Gly Ser Leu Ser Phe Gly Val Ser Glu Leu Met Lys Met Pro Ala Ser	
260 265 270	
gga tgg tat aaa gct cac aac caa gaa gag ggc gaa tat tac aac gtg	864
Gly Trp Tyr Lys Ala His Asn Gln Glu Glu Gly Glu Tyr Tyr Asn Val	
275 280 285	
ccc att cca gaa gga gat gaa gaa ggc aac atg gaa ctc agg cag aag	912
Pro Ile Pro Glu Gly Asp Glu Glu Gly Asn Met Glu Leu Arg Gln Lys	
290 295 300	
ttt gag aaa gcc aag cta ggt cct gtt ggt aac aaa gtc atc agc cct	960
Phe Glu Lys Ala Lys Leu Gly Pro Val Gly Asn Lys Val Ile Ser Pro	
305 310 315 320	
tca gaa gac aga aag caa cca tcc aac aac ctg gac aga gtg aaa ctc	1008
Ser Glu Asp Arg Lys Gln Pro Ser Asn Asn Leu Asp Arg Val Lys Leu	
325 330 335	
aca gac ttc aac ttc ctc atg gtg ctg ggg aag ggg agt ttt ggg aag	1056
Thr Asp Phe Asn Phe Leu Met Val Leu Gly Lys Gly Ser Phe Gly Lys	
340 345 350	
gtg atg ctt gct gac agg aag gga acg gag gaa ctg tac gcc atc aag	1104

Val Met Leu Ala Asp Arg Lys Gly Thr Glu Glu Leu Tyr Ala Ile Lys  
 355 360 365

atc ctg aag aag gac gtg gtg atc cag gac gac gac gtg gag tgc acc 1152  
 Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp Asp Val Glu Cys Thr  
 370 375 380

atg gtg gag aag cgc gtg ctg gcc ctg ctg gac aag ccg cca ttt ctg 1200  
 Met Val Glu Lys Arg Val Leu Ala Leu Leu Asp Lys Pro Pro Phe Leu  
 385 390 395 400

aca cag ctg cac tcc tgc ttc cag aca gtg gac cgg ctg tac ttc gtc 1248  
 Thr Gln Leu His Ser Cys Phe Gln Thr Val Asp Arg Leu Tyr Phe Val  
 405 410 415

atg gaa tac gtc aac ggc ggg gat ctt atg tac cac att cag caa gtc 1296  
 Met Glu Tyr Val Asn Gly Gly Asp Leu Met Tyr His Ile Gln Gln Val  
 420 425 430

ggg aaa ttt aag gag cca caa gca gta ttc tac gca gcc gag atc tcc 1344  
 Gly Lys Phe Lys Glu Pro Gln Ala Val Phe Tyr Ala Ala Glu Ile Ser  
 435 440 445

atc gga ctg ttc ttc ctt cat aaa aga ggg atc att tac agg gat ctg 1392  
 Ile Gly Leu Phe Phe Leu His Lys Arg Gly Ile Ile Tyr Arg Asp Leu  
 450 455 460

aag ctg aac aat gtc atg ctg aac tca gaa ggg cac atc aaa atc gcc 1440  
 Lys Leu Asn Asn Val Met Leu Asn Ser Glu Gly His Ile Lys Ile Ala  
 465 470 475 480

gac ttc ggg atg tgc aag gaa cac atg atg gat gga gtc acg acc agg 1488  
 Asp Phe Gly Met Cys Lys Glu His Met Met Asp Gly Val Thr Thr Arg  
 485 490 495

acc ttc tgc gga act ccg gac tac att gcc cca gag ata atc gct tac 1536  
 Thr Phe Cys Gly Thr Pro Asp Tyr Ile Ala Pro Glu Ile Ile Ala Tyr  
 500 505 510

cag ccg tac ggg aag tct gta gat tgg tgg gcg tac ggt gtg ctg ctg 1584

Gln Pro Tyr Gly Lys Ser Val Asp Trp Trp Ala Tyr Gly Val Leu Leu	
515 520 525	
tac gag atg cta gcc ggg cag cct ccg ttt gat ggt gaa gat gaa gat	1632
Tyr Glu Met Leu Ala Gly Gln Pro Pro Phe Asp Gly Glu Asp Glu Asp	
530 535 540	
gaa ctg ttt cag tct ata atg gag cac aac gtg tcc tac ccc aaa tcc	1680
Glu Leu Phe Gln Ser Ile Met Glu His Asn Val Ser Tyr Pro Lys Ser	
545 550 555 560	
ttg tcc aag gaa gcc gtc tcc atc tgc aaa gga ctt atg acc aaa cag	1728
Leu Ser Lys Glu Ala Val Ser Ile Cys Lys Gly Leu Met Thr Lys Gln	
565 570 575	
cct gcc aag cga ctg ggc tgc ggg ccc gag gga gag agg gat gtc aga	1776
Pro Ala Lys Arg Leu Gly Cys Gly Pro Glu Gly Glu Arg Asp Val Arg	
580 585 590	
gag cat gcc ttc ttc agg agg atc gac tgg gag aaa ctg gag aac agg	1824
Glu His Ala Phe Phe Arg Arg Ile Asp Trp Glu Lys Leu Glu Asn Arg	
595 600 605	
gag atc caa cca cca ttc aag ccc aaa gtg tgt ggc aaa gga gca gaa	1872
Glu Ile Gln Pro Pro Phe Lys Pro Lys Val Cys Gly Lys Gly Ala Glu	
610 615 620	
aac ttt gac aag ttc ttc acg cga gga cag cct gtc tta aca cca cca	1920
Asn Phe Asp Lys Phe Phe Thr Arg Gly Gln Pro Val Leu Thr Pro Pro	
625 630 635 640	
gat cag ctg gtc att gct aac ata gac caa tct gat ttt gaa ggg ttc	1968
Asp Gln Leu Val Ile Ala Asn Ile Asp Gln Ser Asp Phe Glu Gly Phe	
645 650 655	
tcg tat gtc aac ccc cag ttt gtg cac cca atc ttg caa agt gca gta	2016
Ser Tyr Val Asn Pro Gln Phe Val His Pro Ile Leu Gln Ser Ala Val	
660 665 670	
ggg cgc gcc atg agt aaa gga gaa gaa ctt ttc act gga gtt gtc cca	2064

SDOCID &lt;WO 0023615A2 | &gt;

Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Lys Asp  
           835                          840                          845  
  
 gga agc gtt caa tta gca gac cat tat caa caa aat act cca att ggc 2592  
 Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly  
           850                          855                          860  
  
 gat ggc cct gtc ctt tta cca gac aac cat tac ctg tcc acg caa tct 2640  
 Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser  
           865                          870                          875                          880  
  
 gcc ctt tcc aaa gat ccc aac gaa aag aga gat cac atg atc ctt ctt 2688  
 Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Ile Leu Leu  
                           885                          890                          895  
  
 gag ttt gta aca gct gct ggg att aca cat ggc atg gat gaa cta tac 2736  
 Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr  
                           900                          905                          910  
  
 aaa cct cag gag taa 2751  
 Lys Pro Gln Glu \*  
           915

&lt;210&gt; 4

&lt;211&gt; 916

&lt;212&gt; PRT

&lt;213&gt; Aequorea victoria and mouse

&lt;400&gt; 4

Met Ala Asp Val Tyr Pro Ala Asn Asp Ser Thr Ala Ser Gln Asp Val  
   1                          5                          10                          15  
 Ala Asn Arg Phe Ala Arg Lys Gly Ala Leu Arg Gln Lys Asn Val His  
           20                          25                          30  
 Glu Val Lys Asp His Lys Phe Ile Ala Arg Phe Phe Lys Gln Pro Thr  
           35                          40                          45  
 Phe Cys Ser His Cys Thr Asp Phe Ile Trp Gly Phe Gly Lys Gln Gly  
   50                          55                          60  
 Phe Gln Cys Gln Val Cys Cys Phe Val Val His Lys Arg Cys His Glu  
   65                          70                          75                          80

Phe Val Thr Phe Ser Cys Pro Gly Ala Asp Lys Gly Pro Asp Thr Asp  
                     85                    90                    95  
 Asp Pro Arg Ser Lys His Lys Phe Lys Ile His Thr Tyr Gly Ser Pro  
                     100                    105                    110  
 Thr Phe Cys Asp His Cys Gly Ser Leu Leu Tyr Gly Leu Ile His Gln  
                     115                    120                    125  
 Gly Met Lys Cys Asp Thr Cys Asp Met Asn Val His Asn Gln Cys Val  
                     130                    135                    140  
 Ile Asn Asp Pro Ser Leu Cys Gly Met Asp His Thr Glu Lys Arg Gly  
 145                    150                    155                    160  
 Arg Ile Tyr Leu Lys Ala Glu Val Thr Asp Glu Lys Leu His Val Thr  
                     165                    170                    175  
 Val Arg Asp Ala Lys Asn Leu Ile Pro Met Asp Pro Asn Gly Leu Ser  
                     180                    185                    190  
 Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro Lys Asn Glu Ser  
                     195                    200                    205  
 Lys Gln Lys Thr Lys Thr Ile Arg Ser Asn Leu Asn Pro Gln Trp Asn  
                     210                    215                    220  
 Glu Ser Phe Thr Phe Lys Leu Lys Pro Ser Asp Lys Asp Arg Arg Leu  
 225                    230                    235                    240  
 Ser Val Glu Ile Trp Asp Trp Asp Arg Thr Thr Arg Asn Asp Phe Met  
                     245                    250                    255  
 Gly Ser Leu Ser Phe Gly Val Ser Glu Leu Met Lys Met Pro Ala Ser  
                     260                    265                    270  
 Gly Trp Tyr Lys Ala His Asn Gln Glu Glu Gly Glu Tyr Tyr Asn Val  
                     275                    280                    285  
 Pro Ile Pro Glu Gly Asp Glu Glu Gly Asn Met Glu Leu Arg Gln Lys  
                     290                    295                    300  
 Phe Glu Lys Ala Lys Leu Gly Pro Val Gly Asn Lys Val Ile Ser Pro  
 305                    310                    315                    320  
 Ser Glu Asp Arg Lys Gln Pro Ser Asn Asn Leu Asp Arg Val Lys Leu  
                     325                    330                    335  
 Thr Asp Phe Asn Phe Leu Met Val Leu Gly Lys Gly Ser Phe Gly Lys  
                     340                    345                    350  
 Val Met Leu Ala Asp Arg Lys Gly Thr Glu Glu Leu Tyr Ala Ile Lys  
                     355                    360                    365  
 Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp Asp Val Glu Cys Thr  
                     370                    375                    380  
 Met Val Glu Lys Arg Val Leu Ala Leu Leu Asp Lys Pro Pro Phe Leu  
 385                    390                    395                    400



Thr Gln Leu His Ser Cys Phe Gln Thr Val Asp Arg Leu Tyr Phe Val  
 405 410 415  
 Met Glu Tyr Val Asn Gly Gly Asp Leu Met Tyr His Ile Gln Gln Val  
 420 425 430  
 Gly Lys Phe Lys Glu Pro Gln Ala Val Phe Tyr Ala Ala Glu Ile Ser  
 435 440 445  
 Ile Gly Leu Phe Phe Leu His Lys Arg Gly Ile Ile Tyr Arg Asp Leu  
 450 455 460  
 Lys Leu Asn Asn Val Met Leu Asn Ser Glu Gly His Ile Lys Ile Ala  
 465 470 475 480  
 Asp Phe Gly Met Cys Lys Glu His Met Met Asp Gly Val Thr Thr Arg  
 485 490 495  
 Thr Phe Cys Gly Thr Pro Asp Tyr Ile Ala Pro Glu Ile Ile Ala Tyr  
 500 505 510  
 Gln Pro Tyr Gly Lys Ser Val Asp Trp Trp Ala Tyr Gly Val Leu Leu  
 515 520 525  
 Tyr Glu Met Leu Ala Gly Gln Pro Pro Phe Asp Gly Glu Asp Glu Asp  
 530 535 540  
 Glu Leu Phe Gln Ser Ile Met Glu His Asn Val Ser Tyr Pro Lys Ser  
 545 550 555 560  
 Leu Ser Lys Glu Ala Val Ser Ile Cys Lys Gly Leu Met Thr Lys Gln  
 565 570 575  
 Pro Ala Lys Arg Leu Gly Cys Gly Pro Glu Gly Glu Arg Asp Val Arg  
 580 585 590  
 Glu His Ala Phe Phe Arg Arg Ile Asp Trp Glu Lys Leu Glu Asn Arg  
 595 600 605  
 Glu Ile Gln Pro Pro Phe Lys Pro Lys Val Cys Gly Lys Gly Ala Glu  
 610 615 620  
 Asn Phe Asp Lys Phe Phe Thr Arg Gly Gln Pro Val Leu Thr Pro Pro  
 625 630 635 640  
 Asp Gln Leu Val Ile Ala Asn Ile Asp Gln Ser Asp Phe Glu Gly Phe  
 645 650 655  
 Ser Tyr Val Asn Pro Gln Phe Val His Pro Ile Leu Gln Ser Ala Val  
 660 665 670  
 Gly Arg Ala Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro  
 675 680 685  
 Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly Gln Lys Phe Ser Val  
 690 695 700  
 Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys  
 705 710 715 720

16

Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val  
                     725                    730                    735  
 Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His  
                     740                    745                    750  
 Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val  
                     755                    760                    765  
 Gln Glu Arg Thr Ile Phe Tyr Lys Asp Asp Gly Asn Tyr Lys Thr Arg  
                     770                    775                    780  
 Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu  
 785                    790                    795                    800  
 Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Met  
                     805                    810                    815  
 Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Pro  
                     820                    825                    830  
 Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Lys Asp  
                     835                    840                    845  
 Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly  
                     850                    855                    860  
 Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser  
 865                    870                    875                    880  
 Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Ile Leu Leu  
                     885                    890                    895  
 Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr  
                     900                    905                    910  
 Lys Pro Gln Glu  
                     915

&lt;210&gt; 5

&lt;211&gt; 1896

&lt;212&gt; DNA

&lt;213&gt; Aequorea victoria and human

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)...(1896)

&lt;400&gt; 5

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg  
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1                    5                    10                    15

48

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag	240
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag	288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	

gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	
225 230 235 240	
gga ctc aga tct cga gct caa gct tcg aat tca acc atg gcg gcg gcg	768
Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Thr Met Ala Ala Ala	
245 250 255	
gcg gct cag ggg ggc ggg ggc ggg gag ccc cgt aga acc gag ggg gtc	816
Ala Ala Gln Gly Gly Gly Gly Gly Glu Pro Arg Arg Thr Glu Gly Val	
260 265 270	
ggc ccg ggg gtc ccg ggg gag gtg gag atg gtg aag ggg cag ccg ttc	864
Gly Pro Gly Val Pro Gly Glu Val Glu Met Val Lys Gly Gln Pro Phe	
275 280 285	
gac gtg ggc ccg cgc tac acg cag ttg cag tac atc ggc gag ggc gcg	912
Asp Val Gly Pro Arg Tyr Thr Gln Leu Gln Tyr Ile Gly Glu Gly Ala	
290 295 300	
tac ggc atg gtc agc tcg gcc tat gac cac gtg cgc aag act cgc gtg	960
Tyr Gly Met Val Ser Ser Ala Tyr Asp His Val Arg Lys Thr Arg Val	
305 310 315 320	
gcc atc aag aag atc agc ccc ttc gaa cat cag acc tac tgc cag cgc	1008
Ala Ile Lys Lys Ile Ser Pro Phe Glu His Gln Thr Tyr Cys Gln Arg	
325 330 335	

acg ctc cgg gag atc cag atc ctg ctg cgc ttc cgc cat gag aat gtc 1056  
 Thr Leu Arg Glu Ile Gln Ile Leu Leu Arg Phe Arg His Glu Asn Val  
 340 345 350

atc ggc atc cga gac att ctg cgg gcg tcc acc ctg gaa gcc atg aga 1104  
 Ile Gly Ile Arg Asp Ile Leu Arg Ala Ser Thr Leu Glu Ala Met Arg  
 355 360 365

gat gtc tac att gtg cag gac ctg atg gag act gac ctg tac aag ttg 1152  
 Asp Val Tyr Ile Val Gln Asp Leu Met Glu Thr Asp Leu Tyr Lys Leu  
 370 375 380

ctg aaa agc cag cag ctg agc aat gac cat atc tgc tac ttc ctc tac 1200  
 Leu Lys Ser Gln Gln Leu Ser Asn Asp His Ile Cys Tyr Phe Leu Tyr  
 385 390 395 400

cag atc ctg cgg ggc ctc aag tac atc cac tcc gcc aac gtg ctc cac 1248  
 Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asn Val Leu His  
 405 410 415

cga gat cta aag ccc tcc aac ctg ctc agc aac acc acc tgc gac ctt 1296  
 Arg Asp Leu Lys Pro Ser Asn Leu Leu Ser Asn Thr Thr Cys Asp Leu  
 420 425 430

aag att tgt gat ttc ggc ctg gcc cgg att gcc gat cct gag cat gac 1344  
 Lys Ile Cys Asp Phe Gly Leu Ala Arg Ile Ala Asp Pro Glu His Asp  
 435 440 445

cac acc ggc ttc ctg acg gag tat gtg gct acg cgc tgg tac cgg gcc 1392  
 His Thr Gly Phe Leu Thr Glu Tyr Val Ala Thr Arg Trp Tyr Arg Ala  
 450 455 460

cca gag atc atg ctg aac tcc aag ggc tat acc aag tcc atc gac atc 1440  
 Pro Glu Ile Met Leu Asn Ser Lys Gly Tyr Thr Lys Ser Ile Asp Ile  
 465 470 475 480

tgg tct gtg ggc tgc att ctg gct gag atg ctc tct aac cgg ccc atc 1488  
 Trp Ser Val Gly Cys Ile Leu Ala Glu Met Leu Ser Asn Arg Pro Ile  
 485 490 495

ttc cct ggc aag cac tac ctg gat cag ctc aac cac att ctg ggc atc 1536  
 Phe Pro Gly Lys His Tyr Leu Asp Gln Leu Asn His Ile Leu Gly Ile  
 500 505 510

ctg ggc tcc cca tcc cag gag gac ctg aat tgt atc atc aac atg aag 1584  
 Leu Gly Ser Pro Ser Gln Glu Asp Leu Asn Cys Ile Ile Asn Met Lys  
 515 520 525

gcc cga aac tac cta cag tct ctg ccc tcc aag acc aag gtg gct tgg 1632  
 Ala Arg Asn Tyr Leu Gln Ser Leu Pro Ser Lys Thr Lys Val Ala Trp  
 530 535 540

gcc aag ctt ttc ccc aag tca gac tcc aaa gcc ctt gac ctg ctg gac 1680  
 Ala Lys Leu Phe Pro Lys Ser Asp Ser Lys Ala Leu Asp Leu Leu Asp  
 545 550 555 560

cgg atg tta acc ttt aac ccc aat aaa cgg atc aca gtg gag gaa gcg 1728  
 Arg Met Leu Thr Phe Asn Pro Asn Lys Arg Ile Thr Val Glu Glu Ala  
 565 570 575

ctg gct cac ccc tac ctg gag cag tac tat gac ccg acg gat gag cca 1776  
 Leu Ala His Pro Tyr Leu Glu Gln Tyr Tyr Asp Pro Thr Asp Glu Pro  
 580 585 590

gtg gcc gag gag ccc ttc acc ttc gcc atg gag ctg gat gac cta cct 1824  
 Val Ala Glu Glu Pro Phe Thr Phe Ala Met Glu Leu Asp Asp Leu Pro  
 595 600 605

aag gag cgg ctg aag gag ctc atc ttc cag gag aca gca cgc ttc cag 1872  
 Lys Glu Arg Leu Lys Glu Leu Ile Phe Gln Glu Thr Ala Arg Phe Gln  
 610 615 620

ccc gga gtg ctg gag gcc ccc tag 1896  
 Pro Gly Val Leu Glu Ala Pro \*  
 625 630

&lt;210&gt; 6

&lt;211&gt; 631

&lt;212&gt; PRT

&lt;213&gt; Aequorea victoria and human

&lt;400&gt; 6

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1           5           10           15
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
          20           25           30
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
          35           40           45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
          50           55           60
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65           70           75           80
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
          85           90           95
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
          100          105          110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
          115          120          125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
          130          135          140
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145          150          155          160
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
          165          170          175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
          180          185          190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
          195          200          205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
          210          215          220
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
225          230          235          240
Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Thr Met Ala Ala Ala
          245          250          255
Ala Ala Gln Gly Gly Gly Gly Gly Glu Pro Arg Arg Thr Glu Gly Val
          260          265          270
Gly Pro Gly Val Pro Gly Glu Val Glu Met Val Lys Gly Gln Pro Phe
          275          280          285

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Asp Val Gly Pro Arg Tyr Thr Gln Leu Gln Tyr Ile Gly Glu Gly Ala  
 290 295 300  
 Tyr Gly Met Val Ser Ser Ala Tyr Asp His Val Arg Lys Thr Arg Val  
 305 310 315 320  
 Ala Ile Lys Lys Ile Ser Pro Phe Glu His Gln Thr Tyr Cys Gln Arg  
 325 330 335  
 Thr Leu Arg Glu Ile Gln Ile Leu Leu Arg Phe Arg His Glu Asn Val  
 340 345 350  
 Ile Gly Ile Arg Asp Ile Leu Arg Ala Ser Thr Leu Glu Ala Met Arg  
 355 360 365  
 Asp Val Tyr Ile Val Gln Asp Leu Met Glu Thr Asp Leu Tyr Lys Leu  
 370 375 380  
 Leu Lys Ser Gln Gln Leu Ser Asn Asp His Ile Cys Tyr Phe Leu Tyr  
 385 390 395 400  
 Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asn Val Leu His  
 405 410 415  
 Arg Asp Leu Lys Pro Ser Asn Leu Leu Ser Asn Thr Thr Cys Asp Leu  
 420 425 430  
 Lys Ile Cys Asp Phe Gly Leu Ala Arg Ile Ala Asp Pro Glu His Asp  
 435 440 445  
 His Thr Gly Phe Leu Thr Glu Tyr Val Ala Thr Arg Trp Tyr Arg Ala  
 450 455 460  
 Pro Glu Ile Met Leu Asn Ser Lys Gly Tyr Thr Lys Ser Ile Asp Ile  
 465 470 475 480  
 Trp Ser Val Gly Cys Ile Leu Ala Glu Met Leu Ser Asn Arg Pro Ile  
 485 490 495  
 Phe Pro Gly Lys His Tyr Leu Asp Gln Leu Asn His Ile Leu Gly Ile  
 500 505 510  
 Leu Gly Ser Pro Ser Gln Glu Asp Leu Asn Cys Ile Ile Asn Met Lys  
 515 520 525  
 Ala Arg Asn Tyr Leu Gln Ser Leu Pro Ser Lys Thr Lys Val Ala Trp  
 530 535 540  
 Ala Lys Leu Phe Pro Lys Ser Asp Ser Lys Ala Leu Asp Leu Leu Asp  
 545 550 555 560  
 Arg Met Leu Thr Phe Asn Pro Asn Lys Arg Ile Thr Val Glu Glu Ala  
 565 570 575  
 Leu Ala His Pro Tyr Leu Glu Gln Tyr Tyr Asp Pro Thr Asp Glu Pro  
 580 585 590  
 Val Ala Glu Pro Phe Thr Phe Ala Met Glu Leu Asp Asp Leu Pro  
 595 600 605



Lys Glu Arg Leu Lys Glu Leu Ile Phe Gln Glu Thr Ala Arg Phe Gln  
 610 615 620  
 Pro Gly Val Leu Glu Ala Pro  
 625 630

<210> 7

<211> 2160

<212> DNA

<213> Aequorea victoria and human

<220>

<221> CDS

<222> (1)...(2160)

<400> 7

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg 48  
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc 96  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc 144  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc 192  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag 240  
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag 288  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag 336

24

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	
225 230 235 240	
gga ctc aga tct cga gct caa gct tcg aat tcg acc atg tcg tcc atc	768
Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Thr Met Ser Ser Ile	
245 250 255	
ttg cca ttc acg ccg cca gtt gtg aag aga ctg ctg gga tgg aag aag	816

25

Leu Pro Phe Thr Pro Pro Val Val Lys Arg Leu Leu Gly Trp Lys Lys	
260 265 270	
tca gct ggt ggg tct gga gga gca ggc gga gga gag cag aat ggg cag	864
Ser Ala Gly Gly Ser Gly Gly Ala Gly Gly Gly Glu Gln Asn Gly Gln	
275 280 285	
gaa gaa aag tgg tgt gag aaa gca gtg aaa agt ctg gtg aag aag cta	912
Glu Glu Lys Trp Cys Glu Lys Ala Val Lys Ser Leu Val Lys Lys Leu	
290 295 300	
aag aaa aca gga cga tta gat gag ctt gag aaa gcc atc acc act caa	960
Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala Ile Thr Thr Gln	
305 310 315 320	
aac tgt aat act aaa tgt gtt acc ata cca agc act tgc tct gaa att	1008
Asn Cys Asn Thr Lys Cys Val Thr Ile Pro Ser Thr Cys Ser Glu Ile	
325 330 335	
tgg gga ctg agt aca cca aat acg ata gat cag tgg gat aca aca ggc	1056
Trp Gly Leu Ser Thr Pro Asn Thr Ile Asp Gln Trp Asp Thr Thr Gly	
340 345 350	
ctt tac agc ttc tct gaa caa acc agg tct ctt gat ggt cgt ctc cag	1104
Leu Tyr Ser Phe Ser Glu Gln Thr Arg Ser Leu Asp Gly Arg Leu Gln	
355 360 365	
gta tcc cat cga aaa gga ttg cca cat gtt ata tat tgc cga tta tgg	1152
Val Ser His Arg Lys Gly Leu Pro His Val Ile Tyr Cys Arg Leu Trp	
370 375 380	
cgc tgg cct gat ctt cac agt cat cat gaa ctc aag gca att gaa aac	1200
Arg Trp Pro Asp Leu His Ser His His Glu Leu Lys Ala Ile Glu Asn	
385 390 395 400	
tgc gaa tat gct ttt aat ctt aaa aag gat gaa gta tgt gta aac cct	1248
Cys Glu Tyr Ala Phe Asn Leu Lys Lys Asp Glu Val Cys Val Asn Pro	
405 410 415	
tac cac tat cag aga gtt gag aca cca gtt ttg cct cca gta tta gtg	1296

Tyr His Tyr Gln Arg Val Glu Thr Pro Val Leu Pro Pro Val Leu Val  
 420 425 430

ccc cga cac acc gag atc cta aca gaa ctt ccg cct ctg gat gac tat 1344  
 Pro Arg His Thr Glu Ile Leu Thr Glu Leu Pro Pro Leu Asp Asp Tyr  
 435 440 445

act cac tcc att cca gaa aac act aac ttc cca gca gga att gag cca 1392  
 Thr His Ser Ile Pro Glu Asn Thr Asn Phe Pro Ala Gly Ile Glu Pro  
 450 455 460

cag agt aat tat att cca gaa acg cca cct cct gga tat atc agt gaa 1440  
 Gln Ser Asn Tyr Ile Pro Glu Thr Pro Pro Pro Gly Tyr Ile Ser Glu  
 465 470 475 480

gat gga gaa aca agt gac caa cag ttg aat caa agt atg gac aca ggc 1488  
 Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser Met Asp Thr Gly  
 485 490 495

tct cca gca gaa cta tct cct act act ctt tcc cct gtt aat cat agc 1536  
 Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro Val Asn His Ser  
 500 505 510

ttg gat tta cag cca gtt act tac tca gaa cct gca ttt tgg tgt tca 1584  
 Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala Phe Trp Cys Ser  
 515 520 525

ata gca tat tat gaa tta aat cag agg gtt gga gaa acc ttc cat gca 1632  
 Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu Thr Phe His Ala  
 530 535 540

tca cag ccc tca ctc act gta gat ggc ttt aca gac cca tca aat tca 1680  
 Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp Pro Ser Asn Ser  
 545 550 555 560

gag agg ttc tgc tta ggt tta ctc tcc aat gtt aac cga aat gcc acg 1728  
 Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn Arg Asn Ala Thr  
 565 570 575

gta gaa atg aca aga agg cat ata gga aga gga gtg cgc tta tac tac 1776

27

Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val Arg Leu Tyr Tyr	
580	585 590
ata ggt ggg gaa gtt ttt gct gag tgc cta agt gat agt gca atc ttt	1824
Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp Ser Ala Ile Phe	
595 600 605	
gtg cag agc ccc aat tgt aat cag aga tat ggc tgg cac cct gca aca	1872
Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp His Pro Ala Thr	
610 615 620	
gtg tgt aaa att cca cca ggc tgt aat ctg aag atc ttc aac aac cag	1920
Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile Phe Asn Asn Gln	
625 630 635 640	
gaa ttt gct gct ctt ctg gct cag tct gtt aat cag ggt ttt gaa gcc	1968
Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln Gly Phe Glu Ala	
645 650 655	
gtc tat cag cta act aga atg tgc acc ata aga atg agt ttt gtg aaa	2016
Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met Ser Phe Val Lys	
660 665 670	
ggg tgg gga gca gaa tac cga agg cag acg gta aca agt act cct tgc	2064
Gly Trp Gly Ala Glu Tyr Arg Arg Gln Thr Val Thr Ser Thr Pro Cys	
675 680 685	
tgg att gaa ctt cat ctg aat gga cct cta cag tgg ttg gac aaa gta	2112
Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp Leu Asp Lys Val	
690 695 700	
tta act cag atg gga tcc cct tca gtg cgt tgc tca agc atg tca taa	2160
Leu Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser Ser Met Ser *	
705 710 715	

&lt;210&gt; 8

&lt;211&gt; 719

&lt;212&gt; PRT

&lt;213&gt; Aequorea victoria and human

&lt;400&gt; 8

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60  
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240  
 Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Thr Met Ser Ser Ile  
 245 250 255  
 Leu Pro Phe Thr Pro Pro Val Val Lys Arg Leu Leu Gly Trp Lys Lys  
 260 265 270  
 Ser Ala Gly Gly Ser Gly Gly Ala Gly Gly Gly Glu Gln Asn Gly Gln  
 275 280 285  
 Glu Glu Lys Trp Cys Glu Lys Ala Val Lys Ser Leu Val Lys Lys Leu  
 290 295 300

Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala Ile Thr Thr Gln  
 305 310 315 320  
 Asn Cys Asn Thr Lys Cys Val Thr Ile Pro Ser Thr Cys Ser Glu Ile  
 325 330 335  
 Trp Gly Leu Ser Thr Pro Asn Thr Ile Asp Gln Trp Asp Thr Thr Gly  
 340 345 350  
 Leu Tyr Ser Phe Ser Glu Gln Thr Arg Ser Leu Asp Gly Arg Leu Gln  
 355 360 365  
 Val Ser His Arg Lys Gly Leu Pro His Val Ile Tyr Cys Arg Leu Trp  
 370 375 380  
 Arg Trp Pro Asp Leu His Ser His His Glu Leu Lys Ala Ile Glu Asn  
 385 390 395 400  
 Cys Glu Tyr Ala Phe Asn Leu Lys Lys Asp Glu Val Cys Val Asn Pro  
 405 410 415  
 Tyr His Tyr Gln Arg Val Glu Thr Pro Val Leu Pro Pro Val Leu Val  
 420 425 430  
 Pro Arg His Thr Glu Ile Leu Thr Glu Leu Pro Pro Leu Asp Asp Tyr  
 435 440 445  
 Thr His Ser Ile Pro Glu Asn Thr Asn Phe Pro Ala Gly Ile Glu Pro  
 450 455 460  
 Gln Ser Asn Tyr Ile Pro Glu Thr Pro Pro Pro Gly Tyr Ile Ser Glu  
 465 470 475 480  
 Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser Met Asp Thr Gly  
 485 490 495  
 Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro Val Asn His Ser  
 500 505 510  
 Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala Phe Trp Cys Ser  
 515 520 525  
 Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu Thr Phe His Ala  
 530 535 540  
 Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp Pro Ser Asn Ser  
 545 550 555 560  
 Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn Arg Asn Ala Thr  
 565 570 575  
 Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val Arg Leu Tyr Tyr  
 580 585 590  
 Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp Ser Ala Ile Phe  
 595 600 605  
 Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp His Pro Ala Thr  
 610 615 620

## 30

Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile Phe Asn Asn Gln  
 625 630 635 640  
 Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln Gly Phe Glu Ala  
 645 650 655  
 Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met Ser Phe Val Lys  
 660 665 670  
 Gly Trp Gly Ala Glu Tyr Arg Arg Gln Thr Val Thr Ser Thr Pro Cys  
 675 680 685  
 Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp Leu Asp Lys Val  
 690 695 700  
 Leu Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser Ser Met Ser  
 705 710 715

&lt;210&gt; 9

&lt;211&gt; 2157

&lt;212&gt; DNA

<213> *Aequorea victoria* and human

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)...(2157)

&lt;400&gt; 9

atg tcg tcc atc ttg cca ttc acg ccg cca gtt gtg aag aga ctg ctg 48  
 Met Ser Ser Ile Leu Pro Phe Thr Pro Pro Val Val Lys Arg Leu Leu  
 1 5 10 15

gga tgg aag aag tca gct ggt ggg tct gga gga gca ggc gga gga gag 96  
 Gly Trp Lys Lys Ser Ala Gly Gly Ser Gly Gly Ala Gly Gly Gly Glu  
 20 25 30

cag aat ggg cag gaa gaa aag tgg tgt gag aaa gca gtg aaa agt ctg 144  
 Gln Asn Gly Gln Glu Glu Lys Trp Cys Glu Lys Ala Val Lys Ser Leu  
 35 40 45

gtg aag aag cta aag aaa aca gga cga tta gat gag ctt gag aaa gcc 192  
 Val Lys Lys Leu Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala  
 50 55 60

atc acc act caa aac tgt aat act aaa tgt gtt acc ata cca agc act 240





Tyr Ile Ser Glu Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser  
 225 230 235 240  
 atg gac aca ggc tct cca gca gaa cta tct cct act act ctt tcc cct 768  
 Met Asp Thr Gly Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro  
 245 250 255  
 gtt aat cat agc ttg gat tta cag cca gtt act tac tca gaa cct gca 816  
 Val Asn His Ser Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala  
 260 265 270  
 ttt tgg tgt tca ata gca tat tat gaa tta aat cag agg gtt gga gaa 864  
 Phe Trp Cys Ser Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu  
 275 280 285  
 acc ttc cat gca tca cag ccc tca ctc act gta gat ggc ttt aca gac 912  
 Thr Phe His Ala Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp  
 290 295 300  
 cca tca aat tca gag agg ttc tgc tta ggt tta ctc tcc aat gtt aac 960  
 Pro Ser Asn Ser Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn  
 305 310 315 320  
 cga aat gcc acg gta gaa atg aca aga agg cat ata gga aga gga gtg 1008  
 Arg Asn Ala Thr Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val  
 325 330 335  
 cgc tta tac tac ata ggt ggg gaa gtt ttt gct gag tgc cta agt gat 1056  
 Arg Leu Tyr Tyr Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp  
 340 345 350  
 agt gca atc ttt gtg cag agc ccc aat tgt aat cag aga tat ggc tgg 1104  
 Ser Ala Ile Phe Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp  
 355 360 365  
 cac cct gca aca gtg tgt aaa att cca cca ggc tgt aat ctg aag atc 1152  
 His Pro Ala Thr Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile  
 370 375 380  
 ttc aac aac cag gaa ttt gct gct ctt ctg gct cag tct gtt aat cag 1200

Phe Asn Asn Gln Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln  
 385 390 395 400  
 ggt ttt gaa gcc gtc tat cag cta act aga atg tgc acc ata aga atg 1248  
 Gly Phe Glu Ala Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met  
 405 410 415  
 agt ttt gtg aaa ggg tgg gga gca gaa tac cga agg cag acg gta aca 1296  
 Ser Phe Val Lys Gly Trp Gly Ala Glu Tyr Arg Arg Gln Thr Val Thr  
 420 425 430  
 agt act cct tgc tgg att gaa ctt cat ctg aat gga cct cta cag tgg 1344  
 Ser Thr Pro Cys Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp  
 435 440 445  
 ttg gac aaa gta tta act cag atg gga tcc cct tca gtg cgt tgc tca 1392  
 Leu Asp Lys Val Leu Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser  
 450 455 460  
 agc atg tca tgg gta ccg cgg gcc cgg gat cca ccg gtc gcc acc atg 1440  
 Ser Met Ser Trp Val Pro Arg Ala Arg Asp Pro Pro Val Ala Thr Met  
 465 470 475 480  
 gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc 1488  
 Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val  
 485 490 495  
 gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag 1536  
 Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu  
 500 505 510  
 ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc tgc 1584  
 Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys  
 515 520 525  
 acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc ctg 1632  
 Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu  
 530 535 540  
 acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag cag 1680

Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln  
 545 550 555 560  
 cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag cgc 1728  
 His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg  
 565 570 575  
 acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg 1776  
 Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val  
 580 585 590  
 aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc 1824  
 Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile  
 595 600 605  
 gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac aac 1872  
 Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn  
 610 615 620  
 tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac ggc 1920  
 Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly  
 625 630 635 640  
 atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc gtg 1968  
 Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val  
 645 650 655  
 cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc ccc 2016  
 Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro  
 660 665 670  
 gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc 2064  
 Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser  
 675 680 685  
 aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg 2112  
 Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val  
 690 695 700  
 acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag taa 2157

Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys \*  
 705 710 715

<210> 10

<211> 718

<212> PRT

<213> Aequorea victoria and human

<400> 10

Met Ser Ser Ile Leu Pro Phe Thr Pro Pro Val Val Lys Arg Leu Leu  
 1 5 10 15  
 Gly Trp Lys Lys Ser Ala Gly Gly Ser Gly Gly Ala Gly Gly Gly Glu  
 20 25 30  
 Gln Asn Gly Gln Glu Glu Lys Trp Cys Glu Lys Ala Val Lys Ser Leu  
 35 40 45  
 Val Lys Lys Leu Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala  
 50 55 60  
 Ile Thr Thr Gln Asn Cys Asn Thr Lys Cys Val Thr Ile Pro Ser Thr  
 65 70 75 80  
 Cys Ser Glu Ile Trp Gly Leu Ser Thr Pro Asn Thr Ile Asp Gln Trp  
 85 90 95  
 Asp Thr Thr Gly Leu Tyr Ser Phe Ser Glu Gln Thr Arg Ser Leu Asp  
 100 105 110  
 Gly Arg Leu Gln Val Ser His Arg Lys Gly Leu Pro His Val Ile Tyr  
 115 120 125  
 Cys Arg Leu Trp Arg Trp Pro Asp Leu His Ser His His Glu Leu Lys  
 130 135 140  
 Ala Ile Glu Asn Cys Glu Tyr Ala Phe Asn Leu Lys Lys Asp Glu Val  
 145 150 155 160  
 Cys Val Asn Pro Tyr His Tyr Gln Arg Val Glu Thr Pro Val Leu Pro  
 165 170 175  
 Pro Val Leu Val Pro Arg His Thr Glu Ile Leu Thr Glu Leu Pro Pro  
 180 185 190  
 Leu Asp Asp Tyr Thr His Ser Ile Pro Glu Asn Thr Asn Phe Pro Ala  
 195 200 205  
 Gly Ile Glu Pro Gln Ser Asn Tyr Ile Pro Glu Thr Pro Pro Pro Gly  
 210 215 220  
 Tyr Ile Ser Glu Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser  
 225 230 235 240

Met Asp Thr Gly Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro  
 245 250 255  
 Val Asn His Ser Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala  
 260 265 270  
 Phe Trp Cys Ser Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu  
 275 280 285  
 Thr Phe His Ala Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp  
 290 295 300  
 Pro Ser Asn Ser Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn  
 305 310 315 320  
 Arg Asn Ala Thr Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val  
 325 330 335  
 Arg Leu Tyr Tyr Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp  
 340 345 350  
 Ser Ala Ile Phe Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp  
 355 360 365  
 His Pro Ala Thr Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile  
 370 375 380  
 Phe Asn Asn Gln Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln  
 385 390 395 400  
 Gly Phe Glu Ala Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met  
 405 410 415  
 Ser Phe Val Lys Gly Trp Gly Ala Glu Tyr Arg Arg Gln Thr Val Thr  
 420 425 430  
 Ser Thr Pro Cys Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp  
 435 440 445  
 Leu Asp Lys Val Leu Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser  
 450 455 460  
 Ser Met Ser Trp Val Pro Arg Ala Arg Asp Pro Pro Val Ala Thr Met  
 465 470 475 480  
 Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val  
 485 490 495  
 Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu  
 500 505 510  
 Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys  
 515 520 525  
 Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu  
 530 535 540  
 Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln  
 545 550 555 560

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<210> 11
<211> 1908
<212> DNA
<213> Aequorea victoria and human
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<220>  
<221> CDS  
<222> (1)...(1908)
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<400> 11

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg 48

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu

1 5 10 15

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc 96

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly

20 25 30

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc 144

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc 192  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag 240  
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag 288  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag 336  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc 384  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac 432  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140

aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac 480  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160

ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc 528  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175

gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc 576  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190

ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg 624



Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205

agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc 672  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220

gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc 720  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240

gga ctc aga tct cga gct caa gct tcc atg agc gag acg gtc atc atg 768  
 Gly Leu Arg Ser Arg Ala Gln Ala Ser Met Ser Glu Thr Val Ile Met  
 245 250 255

agc gag acg gtc atc tgt tcc agc cgg gcc act gtg atg ctt tat gat 816  
 Ser Glu Thr Val Ile Cys Ser Ser Arg Ala Thr Val Met Leu Tyr Asp  
 260 265 270

gat ggc aac aag cga tgg ctc cct gct ggc acg ggt ccc cag gcc ttc 864  
 Asp Gly Asn Lys Arg Trp Leu Pro Ala Gly Thr Gly Pro Gln Ala Phe  
 275 280 285

agc cgc gtc cag atc tac cac aac ccc acg gcc aat tcc ttt cgc gtc 912  
 Ser Arg Val Gln Ile Tyr His Asn Pro Thr Ala Asn Ser Phe Arg Val  
 290 295 300

gtg ggc cgg aag atg cag ccc gac cag cag gtg gtc atc aac tgt gcc 960  
 Val Gly Arg Lys Met Gln Pro Asp Gln Gln Val Val Ile Asn Cys Ala  
 305 310 315 320

atc gtc cgg ggt gtc aag tat aac cag gcc acc ccc aac ttc cat cag 1008  
 Ile Val Arg Gly Val Lys Tyr Asn Gln Ala Thr Pro Asn Phe His Gln  
 325 330 335

tgg cgc gac gct cgc cag gtc tgg ggc ctc aac ttc ggc agc aag gag 1056  
 Trp Arg Asp Ala Arg Gln Val Trp Gly Leu Asn Phe Gly Ser Lys Glu  
 340 345 350

gat gcg gcc cag ttt gcc gcc ggc atg gcc agt gcc cta gag gcg ttg 1104

Asp Ala Ala Gln Phe Ala Ala Gly Met Ala Ser Ala Leu Glu Ala Leu  
 355 360 365

gaa gga ggt ggg ccc cct cca ccc cca gca ctt ccc acc tgg tcg gtc 1152  
 Glu Gly Gly Gly Pro Pro Pro Pro Pro Ala Leu Pro Thr Trp Ser Val  
 370 375 380

ccg aac ggc ccc tcc ccg gag gag gtg gag cag cag aaa agg cag cag 1200  
 Pro Asn Gly Pro Ser Pro Glu Glu Val Glu Gln Gln Lys Arg Gln Gln  
 385 390 395 400

ccc ggc ccg tcg gag cac ata gag cgc cgg gtc tcc aat gca gga ggc 1248  
 Pro Gly Pro Ser Glu His Ile Glu Arg Arg Val Ser Asn Ala Gly Gly  
 405 410 415

cca cct gct ccc ccc gct ggg ggt cca ccc cca cca cca gga cct ccc 1296  
 Pro Pro Ala Pro Pro Ala Gly Gly Pro Pro Pro Pro Pro Gly Pro Pro  
 420 425 430

cct cct cca ggt ccc ccc cca ccc cca ggt ttg ccc cct tcg ggg gtc 1344  
 Pro Pro Pro Gly Pro Pro Pro Pro Pro Gly Leu Pro Pro Ser Gly Val  
 435 440 445

cca gct gca gcg cac gga gca ggg gga gga cca ccc cct gca ccc cct 1392  
 Pro Ala Ala Ala His Gly Ala Gly Gly Gly Pro Pro Pro Ala Pro Pro  
 450 455 460

ctc ccg gca gca cag ggc cct ggt ggt ggg gga gct ggg gcc cca ggc 1440  
 Leu Pro Ala Ala Gln Gly Pro Gly Gly Gly Gly Ala Gly Ala Pro Gly  
 465 470 475 480

ctg gcc gca gct att gct gga gcc aaa ctc agg aaa gtc agc aag cag 1488  
 Leu Ala Ala Ala Ile Ala Gly Ala Lys Leu Arg Lys Val Ser Lys Gln  
 485 490 495

gag gag gcc tca ggg ggg ccc aca gcc ccc aaa gct gag agt ggt cga 1536  
 Glu Glu Ala Ser Gly Gly Pro Thr Ala Pro Lys Ala Glu Ser Gly Arg  
 500 505 510

agc gga ggt ggg gga ctc atg gaa gag atg aac gcc atg ctg gcc cgg 1584

Ser Gly Gly Gly Gly Leu Met Glu Glu Met Asn Ala Met Leu Ala Arg  
 515 520 525  
 aga agg aaa gcc acg caa gtt ggg gag aaa acc ccc aag gat gaa tct 1632  
 Arg Arg Lys Ala Thr Gln Val Gly Glu Lys Thr Pro Lys Asp Glu Ser  
 530 535 540  
 gcc aat cag gag gag cca gag gcc aga gtc ccg gcc cag agt gaa tct 1680  
 Ala Asn Gln Glu Glu Pro Glu Ala Arg Val Pro Ala Gln Ser Glu Ser  
 545 550 555 560  
 gtg cgg aga ccc tgg gag aag aac agc aca acc ttg cca agg atg aag 1728  
 Val Arg Arg Pro Trp Glu Lys Asn Ser Thr Thr Leu Pro Arg Met Lys  
 565 570 575  
 tcg tct tct tcg gtg acc act tcc gag acc caa ccc tgc acg ccc agc 1776  
 Ser Ser Ser Ser Val Thr Thr Ser Glu Thr Gln Pro Cys Thr Pro Ser  
 580 585 590  
 tcc agt gat tac tcg gac cta cag agg gtg aaa cag gag ctt ctg gaa 1824  
 Ser Ser Asp Tyr Ser Asp Leu Gln Arg Val Lys Gln Glu Leu Leu Glu  
 595 600 605  
 gag gtg aag aag gaa ttg cag aaa gtg aaa gag gaa atc att gaa gcc 1872  
 Glu Val Lys Lys Glu Leu Gln Lys Val Lys Glu Glu Ile Ile Glu Ala  
 610 615 620  
 ttc gtc cag gag ctg agg aag cgg ggt tct ccc tga 1908  
 Phe Val Gln Glu Leu Arg Lys Arg Gly Ser Pro \*  
 625 630 635

&lt;210&gt; 12

&lt;211&gt; 635

&lt;212&gt; PRT

&lt;213&gt; Aequorea victoria and human

&lt;400&gt; 12

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60  
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125  
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr  
 130 135 140  
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn  
 145 150 155 160  
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser  
 165 170 175  
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly  
 180 185 190  
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240  
 Gly Leu Arg Ser Arg Ala Gln Ala Ser Met Ser Glu Thr Val Ile Met  
 245 250 255  
 Ser Glu Thr Val Ile Cys Ser Ser Arg Ala Thr Val Met Leu Tyr Asp  
 260 265 270  
 Asp Gly Asn Lys Arg Trp Leu Pro Ala Gly Thr Gly Pro Gln Ala Phe  
 275 280 285  
 Ser Arg Val Gln Ile Tyr His Asn Pro Thr Ala Asn Ser Phe Arg Val  
 290 295 300  
 Val Gly Arg Lys Met Gln Pro Asp Gln Gln Val Val Ile Asn Cys Ala  
 305 310 315 320  
 Ile Val Arg Gly Val Lys Tyr Asn Gln Ala Thr Pro Asn Phe His Gln  
 325 330 335

Trp Arg Asp Ala Arg Gln Val Trp Gly Leu Asn Phe Gly Ser Lys Glu  
 340 345 350  
 Asp Ala Ala Gln Phe Ala Ala Gly Met Ala Ser Ala Leu Glu Ala Leu  
 355 360 365  
 Glu Gly Gly Gly Pro Pro Pro Pro Pro Ala Leu Pro Thr Trp Ser Val  
 370 375 380  
 Pro Asn Gly Pro Ser Pro Glu Glu Val Glu Gln Gln Lys Arg Gln Gln  
 385 390 395 400  
 Pro Gly Pro Ser Glu His Ile Glu Arg Arg Val Ser Asn Ala Gly Gly  
 405 410 415  
 Pro Pro Ala Pro Pro Ala Gly Gly Pro Pro Pro Pro Pro Gly Pro Pro  
 420 425 430  
 Pro Pro Pro Gly Pro Pro Pro Pro Pro Gly Leu Pro Pro Ser Gly Val  
 435 440 445  
 Pro Ala Ala Ala His Gly Ala Gly Gly Gly Pro Pro Pro Ala Pro Pro  
 450 455 460  
 Leu Pro Ala Ala Gln Gly Pro Gly Gly Gly Gly Ala Gly Ala Pro Gly  
 465 470 475 480  
 Leu Ala Ala Ala Ile Ala Gly Ala Lys Leu Arg Lys Val Ser Lys Gln  
 485 490 495  
 Glu Glu Ala Ser Gly Gly Pro Thr Ala Pro Lys Ala Glu Ser Gly Arg  
 500 505 510  
 Ser Gly Gly Gly Gly Leu Met Glu Glu Met Asn Ala Met Leu Ala Arg  
 515 520 525  
 Arg Arg Lys Ala Thr Gln Val Gly Glu Lys Thr Pro Lys Asp Glu Ser  
 530 535 540  
 Ala Asn Gln Glu Glu Pro Glu Ala Arg Val Pro Ala Gln Ser Glu Ser  
 545 550 555 560  
 Val Arg Arg Pro Trp Glu Lys Asn Ser Thr Thr Leu Pro Arg Met Lys  
 565 570 575  
 Ser Ser Ser Ser Val Thr Thr Ser Glu Thr Gln Pro Cys Thr Pro Ser  
 580 585 590  
 Ser Ser Asp Tyr Ser Asp Leu Gln Arg Val Lys Gln Glu Leu Leu Glu  
 595 600 605  
 Glu Val Lys Lys Glu Leu Gln Lys Val Lys Glu Glu Ile Ile Glu Ala  
 610 615 620  
 Phe Val Gln Glu Leu Arg Lys Arg Gly Ser Pro  
 625 630 635

&lt;211&gt; 2394

&lt;212&gt; DNA

&lt;213&gt; Aequorea victoria and human

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)...(2394)

&lt;400&gt; 13

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg 48  
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu  
 1 5 10 15

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc 96  
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly  
 20 25 30

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc 144  
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile  
 35 40 45

tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc 192  
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr  
 50 55 60

ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag 240  
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys  
 65 70 75 80

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag 288  
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu  
 85 90 95

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag 336  
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu  
 100 105 110

gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc 384  
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly  
 115 120 125

atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	
225 230 235 240	
gga ctc aga tct cga gcc atg gac gaa ctg ttc ccc ctc atc ttc ccg	768
Gly Leu Arg Ser Arg Ala Met Asp Glu Leu Phe Pro Leu Ile Phe Pro	
245 250 255	
gca gag cca gcc cag gcc tct ggc ccc tat gtg gag atc att gag cag	816
Ala Glu Pro Ala Gln Ala Ser Gly Pro Tyr Val Glu Ile Ile Glu Gln	
260 265 270	
ccc aag cag cgg ggc atg cgc ttc cgc tac aag tgc gag ggg cgc tcc	864
Pro Lys Gln Arg Gly Met Arg Phe Arg Tyr Lys Cys Glu Gly Arg Ser	
275 280 285	

gcg ggc agc atc cca ggc gag agg agc aca gat acc acc aag acc cac	912
Ala Gly Ser Ile Pro Gly Glu Arg Ser Thr Asp Thr Thr Lys Thr His	
290 295 300	
ccc acc atc aag atc aat ggc tac aca gga cca ggg aca gtg cgc atc	960
Pro Thr Ile Lys Ile Asn Gly Tyr Thr Gly Pro Gly Thr Val Arg Ile	
305 310 315 320	
tcc ctg gtc acc aag gac cct cct cac cgg cct cac ccc cac gag ctt	1008
Ser Leu Val Thr Lys Asp Pro Pro His Arg Pro His Pro His Glu Leu	
325 330 335	
gta gga aag gac tgc cgg gat ggc ttc tat gag gct gag ctc tgc ccg	1056
Val Gly Lys Asp Cys Arg Asp Gly Phe Tyr Glu Ala Glu Leu Cys Pro	
340 345 350	
gac cgc tgc atc cac agt ttc cag aac ctg gga atc cag tgt gtg aag	1104
Asp Arg Cys Ile His Ser Phe Gln Asn Leu Gly Ile Gln Cys Val Lys	
355 360 365	
aag cgg gac ctg gag cag gct atc agt cag cgc atc cag acc aac aac	1152
Lys Arg Asp Leu Glu Gln Ala Ile Ser Gln Arg Ile Gln Thr Asn Asn	
370 375 380	
aac ccc ttc caa gtt cct ata gaa gag cag cgt ggg gac tac gac ctg	1200
Asn Pro Phe Gln Val Pro Ile Glu Glu Gln Arg Gly Asp Tyr Asp Leu	
385 390 395 400	
aat gct gtg cgg ctc tgc ttc cag gtg aca gtg cgg gac cca tca ggc	1248
Asn Ala Val Arg Leu Cys Phe Gln Val Thr Val Arg Asp Pro Ser Gly	
405 410 415	
agg ccc ctc cgc ctg ccg cct gtc ctt cct cat ccc atc ttt gac aat	1296
Arg Pro Leu Arg Leu Pro Pro Val Leu Pro His Pro Ile Phe Asp Asn	
420 425 430	
cgt gcc ccc aac act gcc gag ctc aag atc tgc cga gtg aac cga aac	1344
Arg Ala Pro Asn Thr Ala Glu Leu Lys Ile Cys Arg Val Asn Arg Asn	
435 440 445	



tct ggc agc tgc ctc ggt ggg gat gag atc ttc cta ctg tgt gac aag	1392
Ser Gly Ser Cys Leu Gly Gly Asp Glu Ile Phe Leu Leu Cys Asp Lys	
450 455 460	
gtg cag aaa gag gac att gag gtg tat ttc acg gga cca ggc tgg gag	1440
Val Gln Lys Glu Asp Ile Glu Val Tyr Phe Thr Gly Pro Gly Trp Glu	
465 470 475 480	
gcc cga ggc tcc ttt tcg caa gct gat gtg cac cga caa gtg gcc att	1488
Ala Arg Gly Ser Phe Ser Gln Ala Asp Val His Arg Gln Val Ala Ile	
485 490 495	
gtg ttc cgg acc cct ccc tac gca gac ccc agc ctg cag gct cct gtg	1536
Val Phe Arg Thr Pro Pro Tyr Ala Asp Pro Ser Leu Gln Ala Pro Val	
500 505 510	
cgt gtc tcc atg cag ctg cgg cgg cct tcc gac cgg gag ctc agt gag	1584
Arg Val Ser Met Gln Leu Arg Arg Pro Ser Asp Arg Glu Leu Ser Glu	
515 520 525	
ccc atg gaa ttc cag tac ctg cca gat aca gac gat cgt cac cgg att	1632
Pro Met Glu Phe Gln Tyr Leu Pro Asp Thr Asp Asp Arg His Arg Ile	
530 535 540	
gag gag aaa cgt aaa agg aca tat gag acc ttc aag agc atc atg aag	1680
Glu Glu Lys Arg Lys Arg Thr Tyr Glu Thr Phe Lys Ser Ile Met Lys	
545 550 555 560	
aag agt cct ttc agc gga ccc acc gac ccc cgg cct cca cct cga cgc	1728
Lys Ser Pro Phe Ser Gly Pro Thr Asp Pro Arg Pro Pro Pro Arg Arg	
565 570 575	
att gct gtg cct tcc cgc agc tca gct tct gtc ccc aag cca gca ccc	1776
Ile Ala Val Pro Ser Arg Ser Ser Ala Ser Val Pro Lys Pro Ala Pro	
580 585 590	
cag ccc tat ccc ttt acg tca tcc ctg agc acc atc aac tat gat gag	1824
Gln Pro Tyr Pro Phe Thr Ser Ser Leu Ser Thr Ile Asn Tyr Asp Glu	
595 600 605	

ttt ccc acc atg gtg ttt cct tct ggg cag atc agc cag gcc tgc gcc	1872
Phe Pro Thr Met Val Phe Pro Ser Gly Gln Ile Ser Gln Ala Ser Ala	
610 615 620	
ttg gcc ccg gcc cct ccc caa gtc ctg ccc cag gct cca gcc cct gcc	1920
Leu Ala Pro Ala Pro Pro Gln Val Leu Pro Gln Ala Pro Ala Pro Ala	
625 630 635 640	
cct gct cca gcc atg gta tca gct ctg gcc cag gcc cca gcc cct gtc	1968
Pro Ala Pro Ala Met Val Ser Ala Leu Ala Gln Ala Pro Ala Pro Val	
645 650 655	
cca gtc cta gcc cca ggc cct cct cag gct gtg gcc cca cct gcc ccc	2016
Pro Val Leu Ala Pro Gly Pro Pro Gln Ala Val Ala Pro Pro Ala Pro	
660 665 670	
aag ccc acc cag gct ggg gaa gga acg ctg tca gag gcc ctg ctg cag	2064
Lys Pro Thr Gln Ala Gly Glu Gly Thr Leu Ser Glu Ala Leu Leu Gln	
675 680 685	
ctg cag ttt gat gat gaa gac ctg ggg gcc ttg ctt ggc aac agc aca	2112
Leu Gln Phe Asp Asp Glu Asp Leu Gly Ala Leu Leu Gly Asn Ser Thr	
690 695 700	
gac cca gct gtg ttc aca gac ctg gca tcc gtc gac aac tcc gag ttt	2160
Asp Pro Ala Val Phe Thr Asp Leu Ala Ser Val Asp Asn Ser Glu Phe	
705 710 715 720	
cag cag ctg ctg aac cag ggc ata cct gtg gcc ccc cac aca act gag	2208
Gln Gln Leu Leu Asn Gln Gly Ile Pro Val Ala Pro His Thr Thr Glu	
725 730 735	
ccc atg ctg atg gag tac cct gag gct ata act cgc cta gtg aca ggg	2256
Pro Met Leu Met Glu Tyr Pro Glu Ala Ile Thr Arg Leu Val Thr Gly	
740 745 750	
gcc cag agg ccc ccc gac cca gct cct gct cca ctg ggg gcc ccg ggg	2304
Ala Gln Arg Pro Pro Asp Pro Ala Pro Ala Pro Leu Gly Ala Pro Gly	
755 760 765	

ctc ccc aat ggc ctc ctt tca gga gat gaa gac ttc tcc tcc att gcg 2352  
 Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser Ile Ala  
 770 775 780

gac atg gac ttc tca gcc ctg ctg agt cag atc agc tcc taa 2394  
 Asp Met Asp Phe Ser Ala Leu Leu Ser Gln Ile Ser Ser \*  
 785 790 795

<210> 14

<211> 797

<212> PRT

<213> Aequorea victoria and human

<400> 14

Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu
1				5					10					15	
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly
			20					25					30		
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile
			35					40					45		
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr
			50				55				60				
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys
65					70				75					80	
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu
					85				90					95	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu
					100				105					110	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly
					115				120					125	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr
					130				135					140	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn
145					150					155				160	
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser
					165				170					175	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly
					180				185					190	

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
 195 200 205  
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe  
 210 215 220  
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser  
 225 230 235 240  
 Gly Leu Arg Ser Arg Ala Met Asp Glu Leu Phe Pro Leu Ile Phe Pro  
 245 250 255  
 Ala Glu Pro Ala Gln Ala Ser Gly Pro Tyr Val Glu Ile Ile Glu Gln  
 260 265 270  
 Pro Lys Gln Arg Gly Met Arg Phe Arg Tyr Lys Cys Glu Gly Arg Ser  
 275 280 285  
 Ala Gly Ser Ile Pro Gly Glu Arg Ser Thr Asp Thr Thr Lys Thr His  
 290 295 300  
 Pro Thr Ile Lys Ile Asn Gly Tyr Thr Gly Pro Gly Thr Val Arg Ile  
 305 310 315 320  
 Ser Leu Val Thr Lys Asp Pro Pro His Arg Pro His Pro His Glu Leu  
 325 330 335  
 Val Gly Lys Asp Cys Arg Asp Gly Phe Tyr Glu Ala Glu Leu Cys Pro  
 340 345 350  
 Asp Arg Cys Ile His Ser Phe Gln Asn Leu Gly Ile Gln Cys Val Lys  
 355 360 365  
 Lys Arg Asp Leu Glu Gln Ala Ile Ser Gln Arg Ile Gln Thr Asn Asn  
 370 375 380  
 Asn Pro Phe Gln Val Pro Ile Glu Glu Gln Arg Gly Asp Tyr Asp Leu  
 385 390 395 400  
 Asn Ala Val Arg Leu Cys Phe Gln Val Thr Val Arg Asp Pro Ser Gly  
 405 410 415  
 Arg Pro Leu Arg Leu Pro Pro Val Leu Pro His Pro Ile Phe Asp Asn  
 420 425 430  
 Arg Ala Pro Asn Thr Ala Glu Leu Lys Ile Cys Arg Val Asn Arg Asn  
 435 440 445  
 Ser Gly Ser Cys Leu Gly Gly Asp Glu Ile Phe Leu Leu Cys Asp Lys  
 450 455 460  
 Val Gln Lys Glu Asp Ile Glu Val Tyr Phe Thr Gly Pro Gly Trp Glu  
 465 470 475 480  
 Ala Arg Gly Ser Phe Ser Gln Ala Asp Val His Arg Gln Val Ala Ile  
 485 490 495  
 Val Phe Arg Thr Pro Pro Tyr Ala Asp Pro Ser Leu Gln Ala Pro Val  
 500 505 510

Arg Val Ser Met Gln Leu Arg Arg Pro Ser Asp Arg Glu Leu Ser Glu  
 515 520 525  
 Pro Met Glu Phe Gln Tyr Leu Pro Asp Thr Asp Asp Arg His Arg Ile  
 530 535 540  
 Glu Glu Lys Arg Lys Arg Thr Tyr Glu Thr Phe Lys Ser Ile Met Lys  
 545 550 555 560  
 Lys Ser Pro Phe Ser Gly Pro Thr Asp Pro Arg Pro Pro Pro Arg Arg  
 565 570 575  
 Ile Ala Val Pro Ser Arg Ser Ser Ala Ser Val Pro Lys Pro Ala Pro  
 580 585 590  
 Gln Pro Tyr Pro Phe Thr Ser Ser Leu Ser Thr Ile Asn Tyr Asp Glu  
 595 600 605  
 Phe Pro Thr Met Val Phe Pro Ser Gly Gln Ile Ser Gln Ala Ser Ala  
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 625 630 635 640  
 Pro Ala Pro Ala Met Val Ser Ala Leu Ala Gln Ala Pro Ala Pro Val  
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 Lys Pro Thr Gln Ala Gly Glu Gly Thr Leu Ser Glu Ala Leu Leu Gln  
 675 680 685  
 Leu Gln Phe Asp Asp Glu Asp Leu Gly Ala Leu Leu Gly Asn Ser Thr  
 690 695 700  
 Asp Pro Ala Val Phe Thr Asp Leu Ala Ser Val Asp Asn Ser Glu Phe  
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 Gln Gln Leu Leu Asn Gln Gly Ile Pro Val Ala Pro His Thr Thr Glu  
 725 730 735  
 Pro Met Leu Met Glu Tyr Pro Glu Ala Ile Thr Arg Leu Val Thr Gly  
 740 745 750  
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 755 760 765  
 Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser Ile Ala  
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&lt;211&gt; 2394

&lt;212&gt; DNA

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<400> 15

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Ile Glu Glu Gln Arg Gly Asp Tyr Asp Leu Asn Ala Val Arg Leu Cys	
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Phe Gln Val Thr Val Arg Asp Pro Ser Gly Arg Pro Leu Arg Leu Pro	
165 170 175	
cct gtc ctt cct cat ccc atc ttt gac aat cgt gcc ccc aac act gcc	576
Pro Val Leu Pro His Pro Ile Phe Asp Asn Arg Ala Pro Asn Thr Ala	
180 185 190	
gag ctc aag atc tgc cga gtg aac cga aac tct ggc agc tgc ctc ggt	624
Glu Leu Lys Ile Cys Arg Val Asn Arg Asn Ser Gly Ser Cys Leu Gly	
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Gly Asp Glu Ile Phe Leu Leu Cys Asp Lys Val Gln Lys Glu Asp Ile	
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Glu Val Tyr Phe Thr Gly Pro Gly Trp Glu Ala Arg Gly Ser Phe Ser	
225 230 235 240	
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Gln Ala Asp Val His Arg Gln Val Ala Ile Val Phe Arg Thr Pro Pro	
245 250 255	
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Tyr Ala Asp Pro Ser Leu Gln Ala Pro Val Arg Val Ser Met Gln Leu	
260 265 270	
cgg cgg cct tcc gac cgg gag ctc agt gag ccc atg gaa ttc cag tac	864
Arg Arg Pro Ser Asp Arg Glu Leu Ser Glu Pro Met Glu Phe Gln Tyr	
275 280 285	
ctg cca gat aca gac gat cgt cac cgg att gag gag aaa cgt aaa agg	912

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ccc acc gac ccc cgg cct cca cct cga cgc att gct gtg cct tcc cgc 1008  
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caa gtc ctg ccc cag gct cca gcc cct gcc cct gct cca gcc atg gta 1200  
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tca gct ctg gcc cag gcc cca gcc cct gtc cca gtc cta gcc cca ggc 1248  
 Ser Ala Leu Ala Gln Ala Pro Ala Pro Val Pro Val Leu Ala Pro Gly  
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cct cct cag gct gtg gcc cca cct gcc ccc aag ccc acc cag gct ggg 1296  
 Pro Pro Gln Ala Val Ala Pro Pro Ala Pro Lys Pro Thr Gln Ala Gly  
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gaa gga acg ctg tca gag gcc ctg ctg cag ctg cag ttt gat gat gaa 1344  
 Glu Gly Thr Leu Ser Glu Ala Leu Leu Gln Leu Gln Phe Asp Asp Glu  
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\*NSDOCID: <WO\_\_\_\_\_0023615A2 | >

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Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr	
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ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac	2064
Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp	
675 680 685	
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Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile	
705 710 715 720	
aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc gtg cag	2208
Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln	
725 730 735	
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Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val	
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ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa	2304
Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys	
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<212> PRT

<213> Aequorea victoria and human

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 Gly Tyr Thr Gly Pro Gly Thr Val Arg Ile Ser Leu Val Thr Lys Asp  
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 Pro Pro His Arg Pro His Pro His Glu Leu Val Gly Lys Asp Cys Arg  
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 Phe Gln Asn Leu Gly Ile Gln Cys Val Lys Lys Arg Asp Leu Glu Gln  
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 Ala Ile Ser Gln Arg Ile Gln Thr Asn Asn Asn Pro Phe Gln Val Pro  
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 145 150 155 160  
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 Pro Val Leu Pro His Pro Ile Phe Asp Asn Arg Ala Pro Asn Thr Ala  
 180 185 190  
 Glu Leu Lys Ile Cys Arg Val Asn Arg Asn Ser Gly Ser Cys Leu Gly  
 195 200 205

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 275 280 285  
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 Pro Pro Gln Ala Val Ala Pro Pro Ala Pro Lys Pro Thr Gln Ala Gly  
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 450 455 460  
 Asp Leu Ala Ser Val Asp Asn Ser Glu Phe Gln Gln Leu Leu Asn Gln  
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 485 490 495  
 Pro Glu Ala Ile Thr Arg Leu Val Thr Gly Ala Gln Arg Pro Pro Asp  
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 Pro Ala Pro Ala Pro Leu Gly Ala Pro Gly Leu Pro Asn Gly Leu Leu  
 515 520 525

Ser Gly Asp Glu Asp Phe Ser Ser Ile Ala Asp Met Asp Phe Ser Ala  
 530 535 540  
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 545 550 555 560  
 Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu  
 565 570 575  
 Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly  
 580 585 590  
 Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr  
 595 600 605  
 Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr  
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 Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His  
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 Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr  
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 Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys  
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 675 680 685  
 Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr  
 690 695 700  
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 755 760 765  
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&lt;211&gt; 2757

&lt;212&gt; DNA

&lt;213&gt; Aequorea victoria and human

&lt;220&gt;

&lt;221&gt; CDS

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1				5					10					15		

gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			

gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
		35					40					45				

tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50				55				60							

ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75					80		

cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85						90				95			

cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
		100						105					110			

gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				

atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
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Ser Asp Lys Asp Arg Arg Leu Ser Val Glu Ile Trp Asp Trp Asp Leu	
485                                      490                                      495	
acc agc agg aat gac ttc atg gga tct ttg tcc ttt ggg att tct gaa	1536
Thr Ser Arg Asn Asp Phe Met Gly Ser Leu Ser Phe Gly Ile Ser Glu	
500                                      505                                      510	
ctt cag aag gcc agt gtt gat ggc tgg ttt aag tta ctg agc cag gag	1584
Leu Gln Lys Ala Ser Val Asp Gly Trp Phe Lys Leu Leu Ser Gln Glu	
515                                      520                                      525	
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Glu Gly Glu Tyr Phe Asn Val Pro Val Pro Pro Glu Gly Ser Glu Ala	
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aat gaa gaa ctg cgg cag aaa ttt gag agg gcc aag atc agt cag gga	1680
Asn Glu Glu Leu Arg Gln Lys Phe Glu Arg Ala Lys Ile Ser Gln Gly	
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Thr Lys Val Pro Glu Glu Lys Thr Thr Asn Thr Val Ser Lys Phe Asp	
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aac aat ggc aac aga gac cgg atg aaa ctg acc gat ttt aac ttc cta	1776
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Met Val Leu Gly Lys Gly Ser Phe Gly Lys Val Met Leu Ser Glu Arg	
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Lys Gly Thr Asp Glu Leu Tyr Ala Val Lys Ile Leu Lys Lys Asp Val	
610                                      615                                      620	



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 Gln Ala Pro Phe Glu Gly Glu Asp Glu Asp Glu Leu Phe Gln Ser Ile  
 785 790 795 800

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 820 825 830

tgt gga cct gaa ggc gaa cgt gat atc aaa gag cat gca ttt ttc cgg 2544  
 Cys Gly Pro Glu Gly Glu Arg Asp Ile Lys Glu His Ala Phe Phe Arg  
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tat att gat tgg gag aaa ctt gaa cgc aaa gag atc cag ccc cct tat 2592  
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 850 855 860

aag cca aaa gct aga gac aag aga gac acc tcc aac ttc gac aaa gag 2640  
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 865 870 875 880

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 Glu Phe Val Ile Asn Val \*  
 915

&lt;210&gt; 18

&lt;211&gt; 918

&lt;212&gt; PRT

&lt;213&gt; Aequorea victoria and human

&lt;400&gt; 18

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Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
      35              40              45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
      50              55              60
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65              70              75              80
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
      85              90              95
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
      100             105             110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115             120             125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130             135             140
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145             150             155             160
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
      165             170             175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
      180             185             190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
      195             200             205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
      210             215             220
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
225             230             235             240
Gly Leu Arg Ser Arg Gly Lys Met Ala Asp Pro Ala Ala Gly Pro Pro
      245             250             255
Pro Ser Glu Gly Glu Glu Ser Thr Val Arg Phe Ala Arg Lys Gly Ala
      260             265             270
Leu Arg Gln Lys Asn Val His Glu Val Lys Asn His Lys Phe Thr Ala
      275             280             285

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 325 330 335  
 Asp Lys Gly Pro Ala Ser Asp Asp Pro Arg Ser Lys His Lys Phe Lys  
 340 345 350  
 Ile His Thr Tyr Ser Ser Pro Thr Phe Cys Asp His Cys Gly Ser Leu  
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 Leu Tyr Gly Leu Ile His Gln Gly Met Lys Cys Asp Thr Cys Met Met  
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 Asp His Thr Glu Arg Arg Gly Arg Ile Tyr Ile Gln Ala His Ile Asp  
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 Met Asp Pro Asn Gly Leu Ser Asp Pro Tyr Val Lys Leu Lys Leu Ile  
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 Pro Asp Pro Lys Ser Glu Ser Lys Gln Lys Thr Lys Thr Ile Lys Cys  
 450 455 460  
 Ser Leu Asn Pro Glu Trp Asn Glu Thr Phe Arg Phe Gln Leu Lys Glu  
 465 470 475 480  
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 485 490 495  
 Thr Ser Arg Asn Asp Phe Met Gly Ser Leu Ser Phe Gly Ile Ser Glu  
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 565 570 575  
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 595 600 605

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 645 650 655  
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 660 665 670  
 Gly Asp Leu Met Tyr His Ile Gln Gln Val Gly Arg Phe Lys Glu Pro  
 675 680 685  
 His Ala Val Phe Tyr Ala Ala Glu Ile Ala Ile Gly Leu Phe Phe Leu  
 690 695 700  
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 Glu Asn Ile Trp Asp Gly Val Thr Thr Lys Thr Phe Cys Gly Thr Pro  
 740 745 750  
 Asp Tyr Ile Ala Pro Glu Ile Ile Ala Tyr Gln Pro Tyr Gly Lys Ser  
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 Met Asn Leu Asp Gln Asn Glu Phe Ala Gly Phe Ser Tyr Thr Asn Pro  
 900 905 910  
 Glu Phe Val Ile Asn Val  
 915

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(19) World Intellectual Property Organization  
International Bureau



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29 March 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR RESPONSE**

(57) Abstract: An improved method and tools for quantifying the effect of an influence on cellular response is described. In particular, an improved method is described for detecting intracellular translocation or redistribution of biologically active polypeptides. The invention also describes several ways of contacting the cells with a substance influencing a cellular response and extracting quantitative information relating to the response in a highly parallel fashion. The method may be used as a very efficient procedure for testing or discovering the influence of a substance on a physiological process using commercially available parallel, high volume assay techniques, for example in connection with screening for new drugs, testing of substances for toxicity, and identifying drug targets for known or novel drugs.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 99/00562

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 G01N33/50 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 96 23898 A (NOVONORDISK AS ; THASTRUP OLE (DK); TULLIN SOEREN (DK); POULSEN LAR) 8 August 1996 (1996-08-08) page 8 -page 17	29-38
X	WO 97 11094 A (NOVONORDISK AS ; THASTRUP OLE (DK); TULLIN SOEREN (DK); POULSEN LAR) 27 March 1997 (1997-03-27) the whole document	29-38
-/-		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

2 February 2000

Date of mailing of the international search report

23. 03. 2000

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/DK 99/00562

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 96 03649 A (UNIV NORTH CAROLINA) 8 February 1996 (1996-02-08) page 49; example 6.10	29-38
X	WO 97 20931 A (US HEALTH ;HTUN HAN (US); HAGER GORDON L (US)) 12 June 1997 (1997-06-12) claims 41-58	29-38
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X	WO 98 30715 A (ISACOFF EHUD Y ;SIEGAL MICAH S (US); UNIV CALIFORNIA (US); CALIFOR) 16 July 1998 (1998-07-16) the whole document	29-38
X	SAKAI ET AL: "Translocation of protein kinase C-gamma and epsilon - Direct visualization in living cells using fusion protein with green fluorescent protein" THE JOURNAL OF CELL BIOLOGY,US,ROCKEFELLER UNIVERSITY PRESS, XP002078902 ISSN: 0021-9525 the whole document	29-38
X	SCHMIDT ET AL: "Dynamic analysis of alpha-PKC-GFP chimera translocation events in smooth muscle with ultra-high speed 3D fluorescence microscopy" FASEB JOURNAL,US,FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, vol. 3, no. 11, page A505 XP002077257 ISSN: 0892-6638 abstract	29-38
	-/-	

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 99/00562

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIDOROVA ET AL: "Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization" MOLECULAR BIOLOGY OF THE CELL,US,BETHESDA, MD, vol. 6, no. 12, page 1641-1658 XP002089512 ISSN: 1059-1524 the whole document	29-38
X	HAN HTUN ET AL: "VISUALIZATION OF GLUCOCORTICOID RECEPTOR TRANSLOCATION AND INTRANUCLEAR ORGANIZATION IN LIVING CELLS WITH A GREEN FLUORESCENT PROTEIN CHIMERA" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 93, no. 10, page 4845-4850 XP002029560 ISSN: 0027-8424 the whole document	29-38
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X	OGAWA H ET AL: "LOCALIZATION, TRAFFICKING, AND TEMPERATURE-DEPENDENCE OF THE AEQUOREA GREEN FLUORESCENT PROTEIN IN CULTURES VERTEBRATE CELLS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 92, no. 25, page 11899-11903 XP002029556 ISSN: 0027-8424 the whole document	29-38
X	WESTPHAL ET AL: "Microfilament dynamics during cell movement and chemotaxis monitored using a GFP - actin fusion protein" CURRENT BIOLOGY,GB,CURRENT SCIENCE,, vol. 7, no. 3, page 176-183 XP002090291 ISSN: 0960-9822 page 181, left-hand column, line 1	29-38
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# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/DK 99/00562

## C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TODA ET AL: "The fission yeast <i>sts5+</i> gene is required for maintenance of growth polarity and functionally interacts with protein kinase C and an osmosensing MAP kinase pathway" JOURNAL OF CELL SCIENCE, GB, ESSEX, vol. 109, no. 9, page 2331-2342 XP002090292 abstract	29-38
X	WEBB ET AL: "Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization during sporulation in <i>Bacillus subtilis</i> " JOURNAL OF BACTERIOLOGY, US, WASHINGTON, DC, vol. 177, no. 20, page 5906-5911 XP002089513 ISSN: 0021-9193 the whole document	29-38
X	WO 94 23039 A (CANCER RES INST ROYAL ; MARSHALL CHRISTOPHER JOHN (GB); ASHWORTH AL) 13 October 1994 (1994-10-13) the whole document	29-38
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK 99/00562

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-28, 39  
because they relate to subject matter not required to be searched by this Authority, namely:  
Rule 39.1(v) PCT - Presentation of information: The subject-matter of claim 39 is a "set of data". This is a mere representation of presentation for which the ISA is not required to establish a search report.
2. ☒ Claims Nos.: 1-28  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-28

Claim 1-28 are not supported by technical terms, as is required by Article 6 and Rule 6.3(a) PCT, which legitimately define the scope of the subject-matter for which protection is sought as no technical contribution to the state of the art commensurate to the scope of the present claims is derivable from the description in terms of a technical problem and a solution thereto as is required by Article 5 and Rule 5.1(iii) PCT. Insofar as claims 1-28 could be understood they would rely on the act of recording of signals from the underlying biological systems and the subsequent processing of the recorded signals. No technical features technically describing such act as a possible contribution to the state of the art is derivable other than the trivial use of state of the art photographic recording devices. No algorithms nor any unexpected combinations of hardware and software defines the subject-matter for which protection is sought. These flaws with respect to the requirements of Article 5 and 6 of the PCT are of such nature that a meaningful complete search could not be executed.

The only technical definition of subject-matter for which a meaningful search could be executed was found in claims limited to the involvement of the technically characterised luminophores as in claims 29-38 and in the parts of the description supporting these claims.

Moreover, the initial phase of the search for this limited subject-matter revealed a very large number of documents relevant to the issue of novelty of claim 1. So many documents were retrieved falling under the wide scope of claim 1-28 that it is impossible to determine which parts of these claim(s) may be said to define subject-matter for which protection might legitimately be sought (Article 6 PCT). For these reasons also, a meaningful search over the whole breadth of the claim(s) is impossible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP comprising luminophore is a polypeptide encoded by SEQ ID No. 1.

2. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP comprising luminophore is a polypeptide encoded by SEQ ID No. 3.

3. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP comprising luminophore is a polypeptide encoded by SEQ ID No. 17

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## 4. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP comprising luminophore is a polypeptide encoded by SEQ ID No. 7.

## 5. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP-comprising luminophore is a polypeptide encoded by SEQ ID No. 9.

## 6. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP comprising luminophore is a polypeptide encoded by SEQ ID No. 11.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## 7. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP comprising luminophore is a polypeptide encoded by SEQ ID No. 13.

## 8. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP comprising luminophore is a polypeptide encoded by SEQ ID No. 15

## 9. Claims: 29-38 IN PART

A method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeabilised living cells, the method comprising recording variation in spatially distributed light emitted from a GFP comprising luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a biochemically active component which is capable of being redistributed in a manner which is related to the degree of influence, as a change in light intensity measured by an instrument designed for the measurement of changes in fluorescence intensity. In which the GFP comprising luminophore is a polypeptide encoded by SEQ ID No. 17



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 99/00562

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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